ARKIV FÖR KEMI Band 11 nr 41

Communicated 13 March 1957 by KARL MYRBÄCK and ARNE FREDGA

Thiophene Chemistry. Part VII

Bromosubstituted thiopheneboronic acids and their diethanolamine esters. The directing effect of the -$B(OH)_2$ group in electrophilic substitution

By SVEN-OLOV LAWESSON

With 5 figures in the text

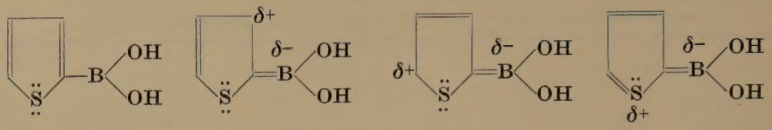
In recent years considerable interest has been shown in the chemistry of the organic boron compounds and intensive research is now being carried out in this field. In connection with work on plant growth regulators currently being carried out at this Institute we have prepared and characterized some aralkylboronic acids (1) which seem to be typical anti-auxines. The aralkylboronic acids are susceptible to oxidation by the air in contrast to the arylboronic acids and therefore we have made an investigation of the latter, partly as to auxine activity and partly as to directing effects.

It is a well-known fact that if a substance is to exhibit auxine activity it must, among other things, have an unsaturated ring system and an acidic side chain. Mostly, carboxylic acids have been investigated but there are reasons to expect the boronic acids to have some activity, too, boron being an oligo element in nature. Some years ago phenylboronic acids were shown to have plant physiological effects (2,3,4,5) and quite recently a reinvestigation has been made by Torssell (6).

In thiophene chemistry very few investigations have been made to determine the ratio of isomeric products when normal meta-directing substituents (in electrophilic substitution) are placed in the 2-or 3-positions respectively. Only two investigations where the entering group is a halogen, have been concerned with the case (7, 8). Almost all experiments showed that the sulfur principally controlled the substitution and that the influence of normally meta-directing groups did not predominate, e.g. a substituent in the 2-position had no influence in most cases. A tendency to form the 4-isomer is always presumed but it is only when the directing group is $-SO_2Cl$ that the entering group preferentially attacks the 4-position (8). When thiophene is substituted by a normal meta-directing group in the 3-position the entering group attacks the 5-position, exclusively.

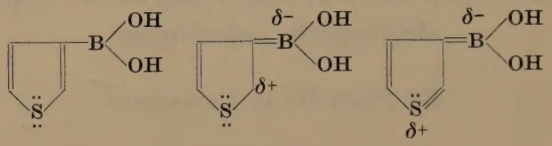
The boronic acid group contains an open sextet of electrons, the boron atom has thus a tendency to accept electrons and it will attract the π-electrons from the aromatic ring. According to the symbolism of Ingold (9) the boronic acid group is characterized as $-I-T$, and the group should thus be meta-directing.

For 2-thiopheneboronic acid the following resonance structures are possible, according to dipole-moment measurements (10) of similar structures.



From these it is seen that if the sulfur had no activating effect, the 4-position, i.e. that meta to the boronic acid group, would first be attacked by an electrophilic reagent.

The following resonance structures are conceivable for 3-thiopheneboronic acid:



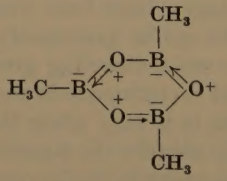
An electron-deficient reagent would enter into the 5-position.

In the thiophene series only 2-thiophene boronic acid has earlier been prepared (11, 12). It was obtained by addition of the Grignard reagent to a trialkyl borate and the hydrolysis of the boronic ester thus formed:

$$RMgX + B(OR')_3 \longrightarrow R{-}B(OR')_2 \xrightarrow{H_2O} R{-}B(OH)_2.$$

This method is general and was first introduced by Khotinsky and Melamed (13). Since that time it has been improved by using the so-called "reversed" reaction and by operating at –60°C (14).

The preparation of Grignard reagents from polybromothiophenes is in most cases impossible but by using an entraining agent the yields will be better (15). If methyl iodide is used, the presence of methylmagnesium iodide in the ethereal solution of the thiophenemagnesium bromide does not prove to be inconvenient in the synthesis of the corresponding thiopheneboronic acid. From the work of Burg (16) it has been shown that methylboronic acid is very unstable and volatible and that it very easily passes over to a trimeric methylboron oxide, with the boiling point 79°C.

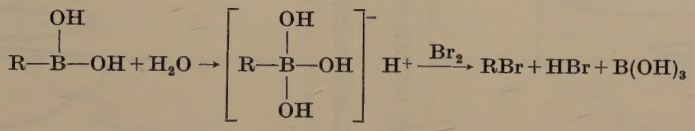


According to this method most bromosubstituted thiophene boronic acids may be obtained and the methylboron oxide formed is vaporized by heating at 80°–90°C. The yields are, however, not high and an alternative procedure for their preparation was investigated.

It has been shown that aromatic lithium compounds are in general much more reactive than the corresponding Grignard reagents (17). Valuable information about the nature and preparation of lithium compounds was obtained from the Lithium

Corporation of America (18). In thiophene lithium chemistry, side reactions predominate if the temperature is not about –60°C (19, 20). As these reagents are so reactive that they cannot be kept over an extended time without the occurrence of side reactions, it is from a practical point of view necessary to use the original method of Khotinsky and Melamed, that is, addition of the butyl borate to the lithium reagent. By adding the butyl borate very quickly there will soon be an excess of borate over lithium reagent and the reaction will be arrested at the boronic acid stage. We have confirmed this and obtained good yields of boronic acids.

In an arylboronic acid it is very easy to replace the boronic acid group by a halogen according to an electrophilic displacement reaction. Water must be present and the following mechanism has been suggested (21).



If the bromination was performed in a water-free medium at a low temperature, nuclear bromination occurred and the acid formed could be isolated in low yields and characterized as its diethanolamine ester. The purity was checked by infrared investigation. The bromination according to Wohl–Ziegler gave better results, however.

The boronic acids are being tested biologically by Professor B. Åberg and the results will be published elsewhere.

Experimental

Reactions with magnesium and lithium were performed under dry oxygen-free nitrogen in a standard Grignard assembly. The infrared spectra were recorded with a Perkin-Elmer spectrophotometer (model 21) with NaCl optics.

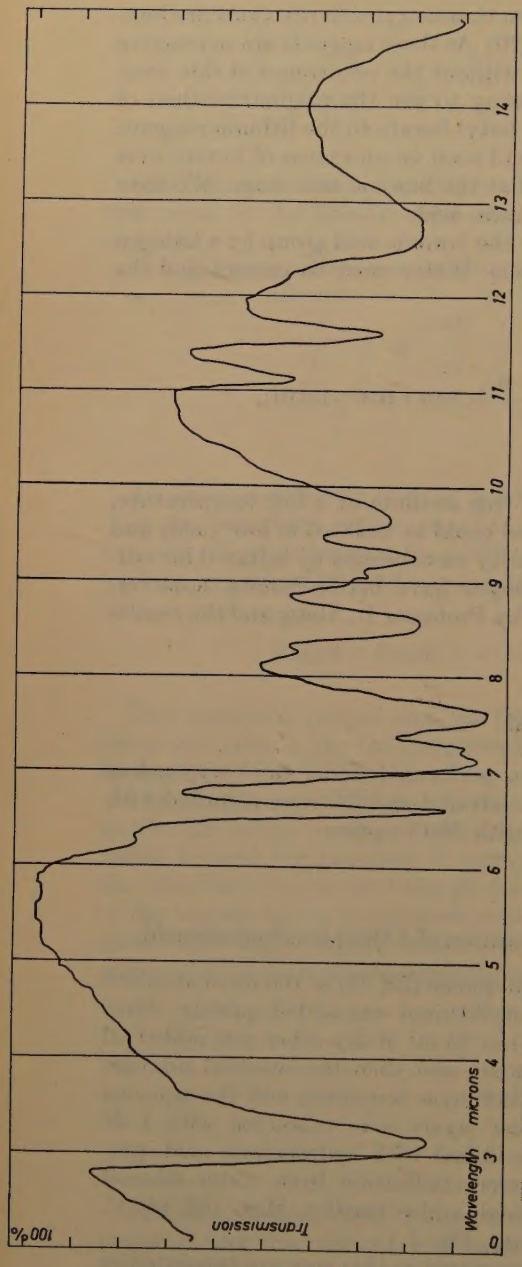
Preparation of thiopheneboronic acids

As a typical example we will describe the preparation of 3-thiopheneboronic acid.

(*a*) *From the lithium reagent.*—5 g of 3-bromothiophene (22, 23) in 100 ml of absolute ether was cooled to –60°C and 30 ml of 1 *M* butyllithium was added quickly. After stirring for 3 minutes, 10 g of butyl borate (24) in 25 ml of dry ether was added all at once. The stirring was continued for four hours and then the reaction mixture was treated with 1 *M* hydrochloric acid, the ether layer separated and the aqueous phase extracted with ether. The combined ether layers were extracted with 1 *M* sodium hydroxide. The alkaline solution was acidified with hydrochloric acid, precipitating the acid as a white powder. After recrystallisation from water–ethanol and drying in air, the acid was obtained as small white needles. M.p. 162–164°C. Weight 2.3 g. Yield 59 %. The infrared spectrum, Fig. 1.

The data of this and the other boronic acids prepared in this way are tabulated in Table 1. The diethanolamine esters were prepared in the usual way (1).

(*b*) *From the Grignard reagent.*—4 g of magnesium was covered with 50 ml of absolute ether. To this a mixture of 4.1 g of 3-bromothiophene and 17.8 g of methyliodide in



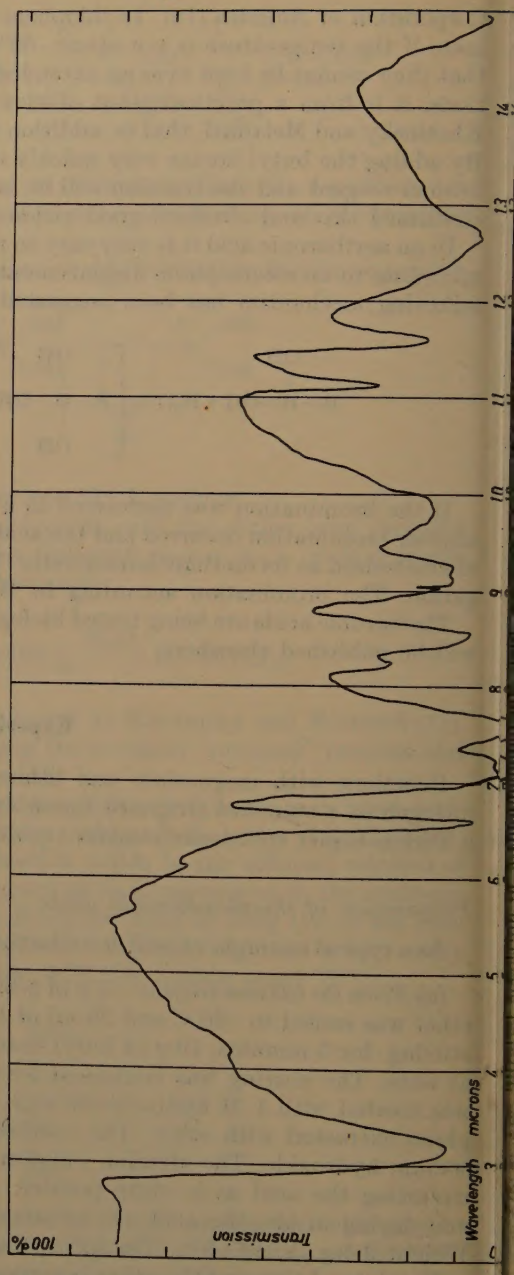


Fig. 1. The infrared spectrum of 3-thiopheneboronic acid, prepared from the lithium reagent (in pressed KBr).

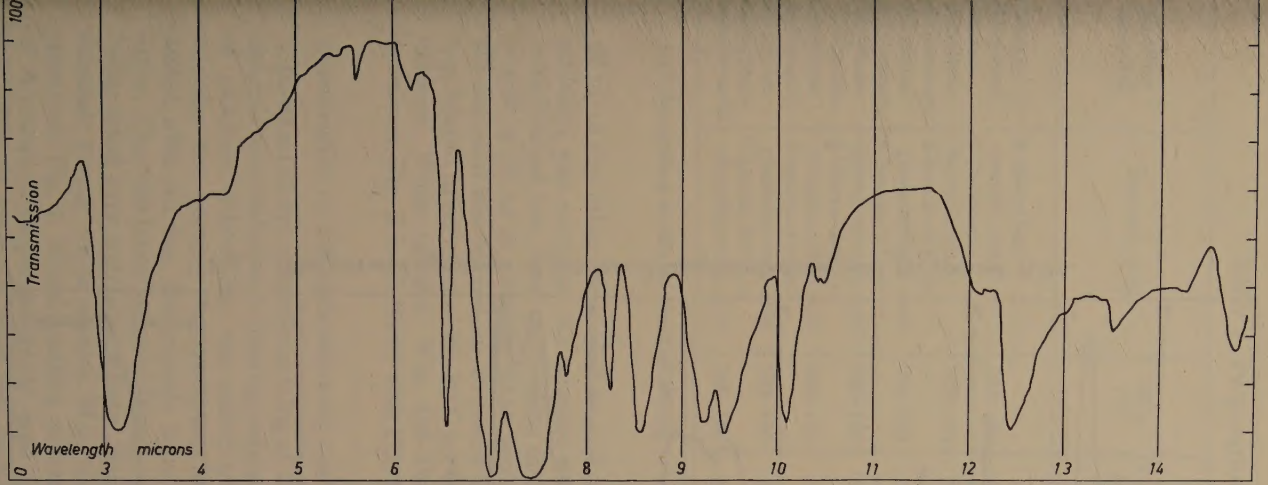


Fig. 3. The infrared spectrum of 5-bromo-2-thiopheneboronic acid, prepared by bromination of 2-thiopheneboronic acid (in pressed KBr).

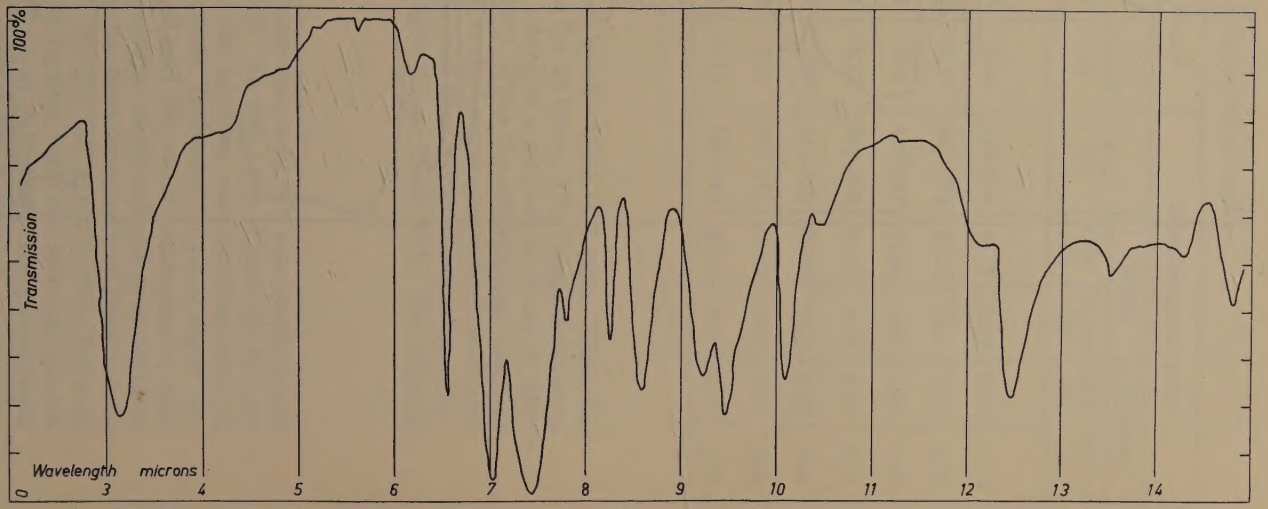


Fig. 4. The infrared spectrum of 5-bromo-2-thiopheneboronic acid prepared from 2,5-dibromothiophene (in pressed KBr).

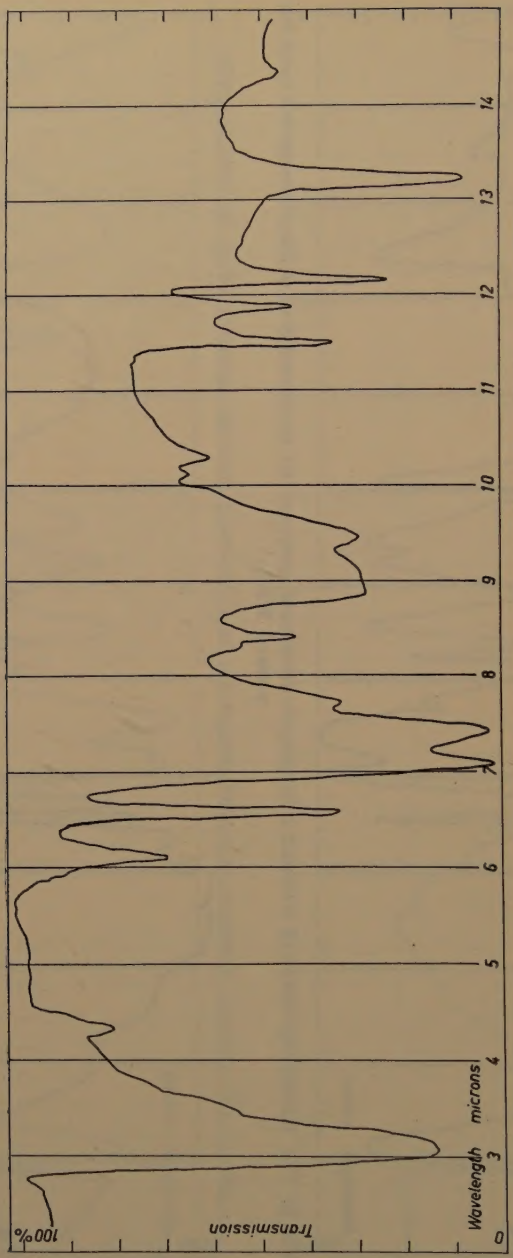


Fig. 5. The infrared spectrum of 4-bromo-2-thiopheneboronic acid (in pressed KBr).

Table 1. Preparation of thiopheneboronic acids.

Starting material	Boronic acid	M.p. (°C)	Yield (%)	Boron		Diethanolamine ester				
				Calc. (%)	Found* (%)	M.p. (°C)	Carbon		Hydrogen	
							Calc. (%)	Found (%)	Calc. (%)	Found (%)
-bromo-thiophene	2-thiophene-boronic acid	132–134	62	8.46	8.50	216–218	48.75	48.4	6.14	6.29
-bromo-thiophene	3-thiophene-boronic acid	162–164	59	8.46	8.53	201–203	48.75	48.1	6.14	6.35
,3-dibromo-thiophene	3-bromo-2-thio-pheneboronic acid	201–203	51	5.23	5.28	229–231	34.82	34.4	4.02	4.20
,4-dibromo-thiophene	4-bromo-2-thio-pheneboronic acid	136–138	56	5.23	5.17	206–208	34.82	34.3	4.02	4.11
5-dibromo-thiophene	5-bromo-2-thio-pheneboronic acid	159–161	58	5.23	5.20	211–213	34.82	34.3	4.02	3.89
,4-dibromo-thiophene	4-bromo-3-thio-pheneboronic acid	193–195	55	5.23	5.27	213–215	34.82	34.5	4.02	3.99
-thiophene-boronic acid	5-bromo-3-thio-pheneboronic acid	128–130	69	5.23	5.16	193–195	34.82	34.4	4.02	4.01

* According to the method of Wittig *et al.* (25).

50 ml of dry ether was added at such a rate that gentle reflux took place. When the addition was complete the mixture was heated for 23 hours. The Grignard reagents were filtered free from magnesium and slowly added to a solution of 45 ml of butyl borate in 40 ml of absolute ether, cooled to –60°C. When the addition was complete the solution was stirred for 10 hours and then worked up as above. The crude acid was heated in an oven at 80–90°C for 30 minutes and then recrystallized from water-ethanol. White needles. M.p. 162–164°C. Weight 0.7 g. Yield 22 %. The infrared spectrum, Fig. 2, is identical with Fig. 1.

Bromination of 2-thiopheneboronic acid

In 60 ml of dry chloroform 0.9 g of 2-thiopheneboronic acid (12) was dissolved and the solution cooled in a freezing-mixture to –40°C. To this 1.2 g of bromine in 5 ml of absolute chloroform was added and when the addition was complete after 20 minutes the stirring was continued for 25 minutes. Thereafter 50 ml of water was added with stirring, the organic layer separated and the water phase shaken with ether. The combined organic layers were evaporated under reduced pressure after being dried over sodium sulphate. The residue was then recrystallized from water–ethanol, giving white needles. M.p. 159–161°C. Weight 0.47 g. Yield 32 %. Infrared spectrum Fig. 3. Mixed melting points and comparison with the infrared spectra of 5-bromo-2-thiopheneboronic acid (Fig. 4) and of 4-bromo-2-thiopheneboronic acid (Fig. 5) revealed, that the bromine had entered the 5-position, exclusively.

Bromination according to Wohl–Ziegler

2-Thiopheneboronic acid (0.9 g) was refluxed in carbon tetrachloride (90 ml) with N-bromosuccinimide (1 g). When the reaction was complete and the insoluble imide

was floating on the surface, the mixture was cooled and filtered. After the solvent had been removed, the residue was recrystallized. White needles. M.p. 159–161°C. Weight 1.1 g. Yield 75 %.

Bromination of 3-thiopheneboronic acid gave in the same way 5-bromo-3-thiopheneboronic acid in a yield of 69 %.

5-Bromo-3-thiopheneboronic acid

To a solution of 1.3 g of 3-thiopheneboronic acid in 120 ml of dry chloroform, cooled to –40°C, 2 g of bromine in 5 ml of absolute chloroform was added. After the addition was complete, stirring was continued for half an hour. The work up was carried out as above. White needles from water–ethanol. M.p. 128–130°C. Weight 0.74 g. Yield 34 %.

Anal. Subst. 15.21 mg; 6.17 ml of 0.1175 *M* NaOH.
$C_4H_4BrO_2SB$ (206.87): Calc. B 5.23.
Found B 5.16.

SUMMARY

By mono-bromination of 2-thiopheneboronic acid 5-bromo-2-thiopheneboronic acid is formed exclusively, i.e. in spite of the meta-directing $-B(OH)_2$ group, the sulfur hetero-atom controls the substitution completely. It is furthermore claimed that 5-bromo-3-thiopheneboronic acid is produced by mono-bromination of 3-thiopheneboronic acid.

The preparation of thiopheneboronic acids is best performed from the lithium reagent. Pure thiopheneboronic acids are also obtained from the Grignard reagents, if methyl iodide is used as entraining agent in the preparation of the latter. By a simple procedure the methylboronic acid (or methylboron oxide) is separated from the mixture.

All monobromothiopheneboronic acids except 2-bromo-3-thiopheneboronic acid have been prepared.

ACKNOWLEDGEMENTS

To Professor A. Fredga I wish to express my sincere gratitude for the facilities he has put at my disposal and for his keen interest in my work. I am indebted to Dr. A. Rosenberg and Mr. S. Bergwall for taking the infrared absorption curves. Thanks are due to Dr. J. Manley for linguistic suggestions in this series of papers.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, March 1957.

REFERENCES

1. LAWESSON, S.-O., Arkiv Kemi *10* (1956), 171.
2. CAUJOLLE, F., and BERGAL, G., Compt. rend. *228* (1949), 1516.
3. CAUJOLLE, F., and Bergal, G., ibid. *230* (1950), 1101.
4. CAUJOLLE, F., and BERGAL, G., ibid. *231* (1950), 1550.
5. CAUJOLLE, F., and BERGAL, G., ibid. *232* (1951), 109.
6. TORSSELL, K., Physiol. Plant. *9* (1956), 652.
7. HARTOUGH, H. D., unpublished.
8. STEINKOPF, W., and HÖPNER, T., Ann. *501* (1933), 174.

9. INGOLD, C. K., Structure and Mechanism in Organic Chemistry. Cornell University Press, Ithaca, New York, 1953.
10. KESWANI, R., and FREISER, H., J. Am. Chem. Soc. *71* (1948), 1789.
11. KRAUSE, E., and RENWANZ, G., Ber. *65* (1932), 777.
12. JOHNSON, J. R., VAN CAMPEN, Jr., M. G., and Grummit, O., J. Am. Chem. Soc. *60* (1938), 111.
13. KHOTINSKY, E., and MELAMED, M., Ber. *42* (1909), 3090.
14. BEAN, F. R., and JOHNSON, J. R., J. Am. Chem. Soc. *54* (1932), 4415.
15. LAWESSON, S.-O., Arkiv Kemi *11* (1957), 337.
16. BURG, A. B., J. Am. Chem. Soc. *62* (1940), 2228.
17. SWAIN, C. G., ibid. *69* (1947), 2306.
18. *Lithium Corporation of America, Inc., Rand Tower, Minneapolis 2, Minnesota*: Organolithium Compounds in Organic Synthesis, 1949. Suppl. No. 1, 1950; Suppl. No. 2, 1952; Suppl. No. 3, 1954; Suppl. No. 4, 1956.
19. LAWESSON, S.-O., Arkiv Kemi *11* (1957), 325.
20. LAWESSON, S.-O., ibid. *11*, (1957), 317.
21. KUIVILA, H. G., and EASTERBROOK, E. K., J. Am. Chem. Soc. *73* (1951), 4629.
22. LAWESSON, S.-O., Arkiv Kemi *11* (1957), 373.
23. LAWESSON, S.-O., Acta Chem. Scand. *10* (1956), 1020.
24. Organic Syntheses, Coll. Vol. III, p. 195.
25. WITTIG, G., KEICHER, G., RÜCKERT, A., and RAFF, P., Ann. *563* (1949), 110.

Tryckt den 24 juni 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 42

Communicated 13 March 1957 by Karl Myrbäck and Arne Fredga

Molecular orbital calculations on the influence of a –I–M substituent on the reactivity of thiophene

By Lars Melander

With 3 figures in the text

Introduction

Some earlier calculations on the reactivity of thiophene (1, 2) and 2-nitrothiophene (3) were based on the thiophene model introduced by Longuet-Higgins (4). In the case of 2-nitrothiophene, Wheland's parameters for the nitro group (5) were used. All these calculations were carried through according to the molecular orbital method (MO LCAO).

The interesting paper of Gronowitz and Rosenberg on the electronic structure of the thiophenecarboxylic acids and the thiophenecarboxaldehydes (6) made it of interest to look for the molecular orbital counterpart to their discussion in terms of resonance structures. Especially their statement that a –M (Ingold's terminology) substituent in position 3 of thiophene should not deactivate position 4 towards electrophilic substitution, contrary to what happens in benzene, seems worth further investigation.

In the present calculations, the nitro group was chosen as a representative of –I–M groups. With a fairly simple and reasonable choice of parameters, it can be made meta-directing with deactivation of the whole nucleus in the case of nitrobenzene, the ortho-substitution being the main side-reaction, all provided that the localisation energy is studied (7). In the beginning of the calculations, the parameters used by Wheland in his first studies of the localisation energy in aromatic substitution (5) were tried, but a numerical error was found in Wheland's localisation energies for the ortho position in nitrobenzene. Thus, the localisation energies for electrophilic substitution in the ortho, meta, and para positions should be 1.846, 1.852 and 1.861 units, respectively, which means that this set of parameters is in reality inappropriate for a group known to be meta-directing and which deactivates the benzene nucleus as a whole. The set of parameters used recently by Matlow and Wheland (7) is, however, very good for nitrobenzene, giving the right order of reactivity to the different positions and one benzene position. This latter set of parameters was used in the calculations reported in the present paper.

It is believed that the results obtained with the parameters of the nitro group are fairly representative also for other substituents of the type –I–M, at least for isoelectronic groups like the carboxyl, notwithstanding that the latter has two non-equivalent oxygen atoms. The orienting effect of all these groups in the corresponding

benzene derivatives is very much the same, the reactivity towards electrophilic reagents decreasing in the order meta > ortho > para (8).

The general connections between the reactivity of conjugated systems and the magnitudes which may be obtained by simple quantum mechanical calculations have been reviewed, for instance by Pullman and Pullman (9) and by R. D. Brown (10) and need no further comments. If a charge approaches position r in a conjugated system, it is reasonable to assume that the coulomb integral α_r of the corresponding carbon atom will change with an amount $\delta\alpha_r$. This will cause an energy change δE in the conjugated system

$$\delta E = \frac{\partial E}{\partial \alpha_r}\delta\alpha_r + \tfrac{1}{2}\frac{\partial^2 E}{\partial \alpha_r^2}(\delta\alpha_r)^2 + \cdots = q_r\,\delta\alpha_r + \tfrac{1}{2}\pi_{rr}(\delta\alpha_r)^2 + \cdots,$$

where q_r is the charge and π_{rr} the self-polarisability of the same atom.

When discussing the relative energetics of different positions in the early stages of a reaction with a charged species, it is generally adequate to study only the charges, provided they are not too similar in magnitude. The self-polarisabilities have not been computed in the present investigation. It is also believed that the free valence (or what Brown (10) calls the localisability effect) is of inferior importance in the early stages, because in most electrophilic substitutions, which are of primary interest, the attacking entity is a cation or at least a positively charged part of a complex, and the unperturbed aromatic derivative carries fairly different charges in different positions.

The localisation energy, i.e. the difference in π-electron energy between the conjugated system which remains after the appropriate number of electrons have been completely localised at the position in question (for the creation of a new σ-bond) and the initial conjugated system, corresponds to a reaction stage, which for instance in nitration is subsequent to the transition state and is very important in the present investigation. Thus, the choice of the parameters was largely determined by their ability to give reasonable localisation energies in the benzene derivative, as mentioned above.

It seems well justified that the primary interest should be attached to the localisation energy, because the corresponding structure, which was formerly considered more or less hypothetical and idealised, seems now likely to be realised in the intermediate of the substitution. A great deal of experimental and theoretical evidence is now in favour of its existence also with simple benzene derivatives in electrophilic substitutions (11–16). In his paper referred to above (10), Brown considers the structure with complete localisation corresponding to a "hypothetical extrapolation of the energy curve". It seems more likely, however, that it is represented by the point *d on* the curve as in Fig. 1. The other points *a*, *b*, and *c* represent, as in Brown's curve, the initial state, an early stage of reaction, e.g. when the coulomb integral α_r has changed to $\alpha_r + \delta\alpha_r$ and the energy from E to $E + \delta E$ as above, and the transition state, respectively. Probably *d* is not situated very far from *c*, and thus the localisation energy should be very significant for the energy level at the transition state. In some cases, to which nitration (11, 12) and at least a special kind of halogenation (13) do not belong, the second maximum might be the higher one, thus corresponding to the transition state. Even in such a case the localisation energy should be significant.

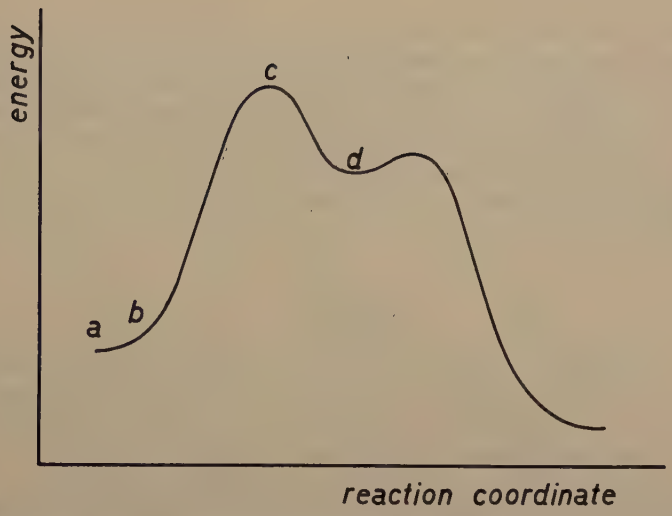


Fig. 1. The potential energy in aromatic substitution as a function of the reaction coordinate. For explanation of the symbols, see the text.

Calculations and results

As mentioned above, the set of parameters given by Matlow and Wheland (7) were used for the nitro group, and the calculations were carried through with the inclusion of overlap in the same way as in the related calculations (4, 5, 7). The parameter x denotes the assumed ratio between a C–S and a C–C resonance or overlap integral. Three values of x were used, $x = 1$, which corresponds to the ordinary nitrobenzene molecule, $x = 0.8$, which corresponds to an earlier value of the resonance energy of thiophene (30 kcal/mol), and $x = 0.6$, which corresponds to the recent value of the resonance energy given by Sunner (20 kcal/mol) (17).

The charges in the different positions and the few bond orders given, have been calculated by means of the formulae including overlap given by Chirgwin and Coulson (18).

All localisation energies are given in Wheland's $-\beta_0$, a positive quantity equivalent to about 38 kcal/mol.

The localisation energies for unsubstituted thiophene have been calculated earlier (2), and its charges are unity, since, according to theory, the compound belongs to the category of alternant conjugated hydrocarbons.

The hyperconjugation between the carbon–hydrogen bond at the reaction centre of the intermediate and the residual π-electron system is likely to be of importance for the localisation energy. This is the case especially in the hydrogen exchange reaction, where the intermediate will contain two sp^3 carbon–hydrogen bonds at the reaction centre (15). For alternant hydrocarbons, however, the inclusion of hyperconjugation does not change the sequence of reactivity (19, 20). Although it is unknown whether the same holds for compounds of the present kind, hyperconjugation has not been included in the calculations.

The localisation energies are given at the respective positions in Fig. 2. In order to facilitate comparison, nitrobenzene occurs twice in the scheme, once with 2-nitro-

and once with 3-nitrothiophene. Since for both kinds of position in thiophene, the orbital containing 0, 1 or 2 electrons for electrophilic, radical, and nucleophilic substitution, respectively, is non-bonding, all these kinds of substitution will give rise to the same localisation energies and could be represented by a single column.

Fig. 3 shows the π-electron distribution in units of the electronic charge. For the same reason as in Fig. 2 nitrobenzene occurs twice.

The calculated π-bond orders for the carbon-nitrogen and the nitrogen-oxygen bonds are given in Table 1.

Discussion

The experimental facts referred to below are conveniently discussed in the paper by Gronowitz and Rosenberg (6) and in the book by Hartough (21), in both of which references to original papers can be found. Experimental facts mentioned in the following without particular reference are found in (6) or (21).

It is immediately evident from Figs. 2 and 3 that the parameters of Matlow and Wheland (7) are good for nitrobenzene only in so far as the localisation energy is considered decisive for the reactivity. The charge distribution tends to make the reactivity towards electrophilic reagents decrease in the order meta $\approx$ benzene $>$ para $>$ ortho. It is quite possible, however, that the π-electron energy curves will cross over, and it has already been stressed in the introduction that it seems natural to ascribe the greatest importance to the localisation energy. Moreover, recent discussions of independent experimental facts (22) seem to lead to the conclusion that the initial charge distribution e.g. in nitrobenzene has little to do with the orientation.

In a previous paper (3), the orientation of a substituent entering the nucleus of 2-nitrothiophene was investigated by means of Wheland's old set of parameters (5). A study of the localisation energies at the positions 4 and 5 showed that any kind of attack could be expected to be directed towards position 5, the directing influence of the sulphur atom being by far the strongest one, even when x was as large as 0.8. According to Fig. 2, the same holds for the new set of parameters, the change causing only minor deviations in the figures.

On the whole, the nitro group in position 2 might be said to have the same influence on the localisation energies for electrophilic substitution in thiophene as in benzene, deactivating the whole nucleus but to the largest extent the ortho and para positions, just as Gronowitz and Rosenberg found in their discussion of resonance structures.

The calculated electronic charge distribution (Fig. 3) shows that the position meta relative to the nitro group carries the highest charge in 2-nitrothiophene as well as in nitrobenzene. Experimental facts again speak against the reactivity being judged from the unperturbed charge distribution, for it is known that the nitro group or the isoelectronic carboxyl group in position 2 cannot outweigh the activation of position 5 by the sulphur atom for electrophilic substitution. With an aldehyde group in position 2 things are different, and the next substituent often prefers position 4. This interesting difference deserves further theoretical investigation. Groups of the latter type, structurally related to the vinyl group, have not been treated in the present investigation.

If the effect of a nitro group in the position 3 of thiophene on the electrophilic reactivity were analogous to that in benzene, the whole nucleus should still be deacti-

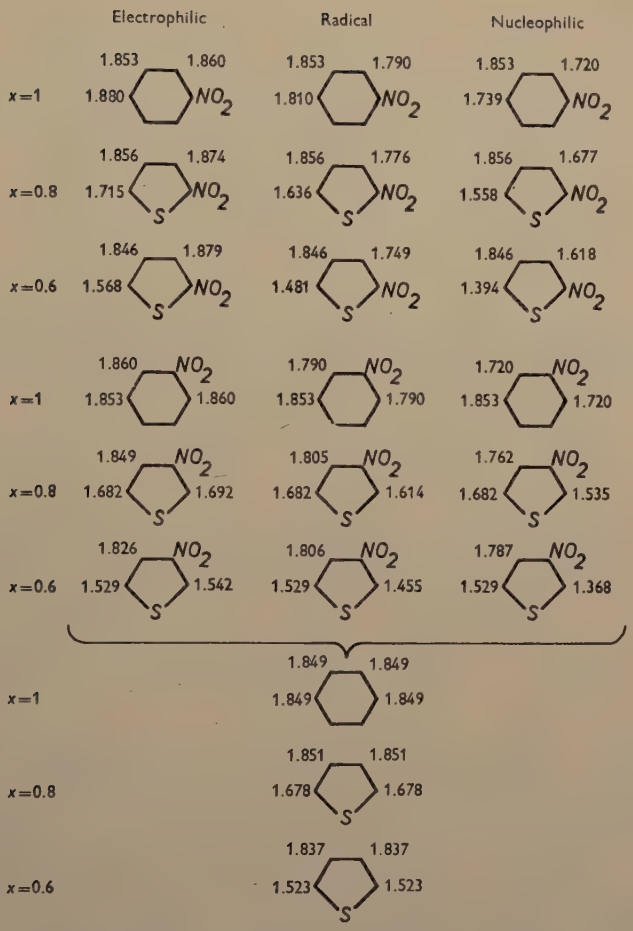


Fig. 2. Calculated localisation energies for nitrobenzene and the nitrothiophenes. Unit is Wheland's $-\beta_0$ [5]. The type of substitution is given at the head of each column.

vated, position 5 slightly and positions 2 and 4 strongly. The values in Fig. 2 show however, that, as far as localisation energies are concerned, this expectation is not entirely correct. Positions 2 and 5 behave similarly as in the benzene derivative but position 4 is in fact activated by the presence of the neighbouring nitro group. The order of reactivity is $5 > 2 \gg 4$, and the last position in 3-nitrothiophene is somewhat more reactive than the corresponding position in thiophene. This agrees very well with the conclusions of Gronowitz and Rosenberg from a consideration of resonance structures and also with several pieces of experimental evidence.

The calculated electronic charges in Fig. 3 would give the order of electrophilic reactivity $5 > 4 > 2$, the unsubstituted thiophene falling between 5 and 4. The order of reactivity given by the localisation energies also in this case seems to give the best agreement with experimental facts.

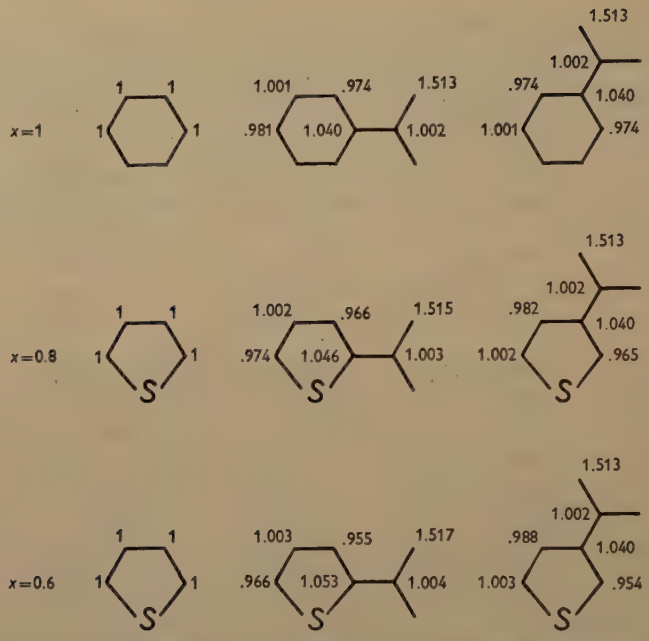


Fig. 3. Calculated π-electron distribution in nitrobenzene and the nitrothiophenes in units of electronic charge.

A few data given by Hurd and Kreuz (23) allow a rough comparison of predicted and experimental reactivities for the case of nucleophilic displacement of chlorine. (The present theoretical results are applicable to this kind of reaction only if effects due to the participation of the chlorine in the π-electron system of the initial molecule are negligible or happen to cancel, which is far from certain.) Unfortunately the experimental results are contradictory. 3-Nitro-2-chlorothiophene is more reactive towards piperidine than is 5-nitro-2-chlorothiophene, which agrees with the localisation energies. In alcoholysis, however, the order of reactivity is reversed, although the difference is not large in the latter reaction. The fact that the same difference in behaviour towards the two reagents occurs also with the corresponding benzene derivatives shows that more detailed information about the mechanism is required for a discussion of the reactivities in these reactions. The rough conclusion that thiophene positions adjacent to sulphur should be more reactive than positions in corresponding benzene compounds is, however, in agreement with observations on 3,5-dinitro-2-chlorothiophene and 2,4-dinitrochlorobenzene.

Gronowitz and Rosenberg conclude from resonance structures that the conjugation between the thiophene nucleus and the substituent should be more extensive for the 2- than the 3-position. This could be anticipated from the calculations of de Heer on the simple thiophene molecule (1). He finds the conjugating power, which is equal to the square root of the self-polarisability, to be 0.672, 0.632, and 0.631 units for the 2- and 3-positions in thiophene ($x = 0.8$) and a position in benzene, respectively. According to Coulson and de Heer, the π-bond order of a bond between two residues is proportional to these conjugating powers of each of the two finally joined atoms in

Table 1. Calculated π-bond orders in nitrobenzene and the nitrothiophenes.

Compound ...	2-nitro-		3-nitro-	
Bond ...	C–N	N–O	C–N	N–O
$x = 1$	.233	.686	.233	.686
$x = 0.8$	.246	.684	.232	.686
$x = 0.6$	.258	.681	.232	.686

their respective residues (24). With one and the same group, the three positions should give bond orders in the approximate ratio $0.672:0.632:0.631 = 1.06:1.00:1$. According to Table 1, the calculated bond orders are also in the ratio $1.06:1.00:1$, in good agreement with the approximate calculation.

The π-bond order of the nitrogen-oxygen bonds has also been calculated. It decreases slightly with decreasing x in the 2-nitro derivative and stays constant in the 3-nitro derivative, thus making it higher in the latter than in the former. The small difference deserves little consideration, especially as the interrelation between bond order and force constant also involves the self-polarisability of the bond in question (25). This magnitude has not been computed in the present investigation, making a direct comparison with the infra-red measurements of Gronowitz and Rosenberg impossible at present.

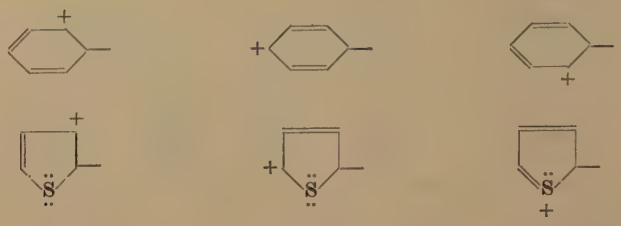
The generally good agreement between experimental results and predictions in the present and previous theoretical investigations (1, 2, 4) may be considered as a good support for the thiophene model introduced by Longuet-Higgins (4). It might be of interest to look for the connection between it and usual reasoning in terms of valence-bond structures.

The study of valence-bond structures by Gronowitz and Rosenberg (6) shows that very reasonable results are obtained without the inclusion of structures with an electron decet around the sulphur. Decet structures involve one or two double bonds between sulphur and carbon. If the Longuet-Higgins model is real, valence bond structures with π-bonds between carbon and sulphur should be assigned low weights, since owing to decreased overlap in these bonds they should be energetically unfavourable.

There are also structures with π-bonds to sulphur but with only an octet around the sulphur. They place a positive charge on the sulphur. According to the present principles, they should be regarded unstable for the same reason as those with a decet, because it is the presence of valence-bonds where overlap is decreased and not the mere presence of a decet that makes a structure energetically unfavourable. Such structures occur in the paper of Gronowitz and Rosenberg, but the conclusions are not changed by assigning them a low weight.

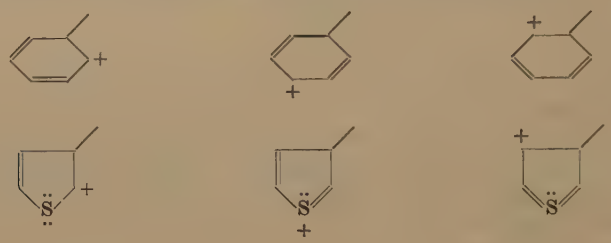
A simple example will show how a study of valence-bond structures according to these principles will reveal the difference in reactivity in electrophilic substitution between the two positions in thiophene. Again the energy of the intermediate with localised electrons at the reaction centre will be studied. For comparison, the corresponding structures in benzene substitution are shown above those for thiophene. The localised electron pair is represented by a valence bond extending outside the ring.

In electrophilic substitution in position 2, the following structures are taken into account:



(A) (B) (C)

For electrophilic substitution in position 3, the structures are:



(D) (E) (F)

Of these structures (C) and (E) have one and (F) has two π-bonds between sulphur and carbon. It is consequently obvious that the intermediate corresponding to substitution in position 2 is the most stable.

Obviously, the last valence-bond scheme does not indicate that benzene, with its three "good" valence bond structures in the intermediate, reacts with greater ease than thiophene. For such a comparison between different compounds the stability of the initial molecules has also to be considered.

SUMMARY

Molecular orbital (MO LCAO) calculations similar to those of Wheland [5] have been carried out on 2- and 3-nitrothiophene using the thiophene model of Longuet-Higgins [4] and the parameters for the nitro group chosen by Matlow and Wheland [7]. The reactivity has been discussed in terms of localisation energies and electronic charges and compared with experimental evidence. The agreement between experimental reactivity and that predicted by localisation energies is good.

The interpretation of the Longuet-Higgins thiophene model in terms of qualitative valence-bond theory is briefly discussed.

The writer has had several interesting and valuable discussions with Fil. lic. Salo Gronowitz, Uppsala. Fil. kand. Stig Olsson of this institute has kindly checked some of the preliminary calculations. The eigenvalues and eigenvectors have been computed at the Swedish digital computor BESK, Stockholm.

Nobel Institute of Chemistry, Stockholm 50, Sweden.

REFERENCES

1. J. DE HEER, J. Am. Chem. Soc. *76* (1954), 4802.
2. L. MELANDER, Arkiv Kemi *8* (1956), 361.
3. L. MELANDER, Acta Chem. Scand. *9* (1955), 1400.
4. H. C. LONGUET-HIGGINS, Trans. Faraday Soc. *45* (1949), 173.
5. G. W. WHELAND, J. Am. Chem. Soc. *64* (1942), 900.
6. S. GRONOWITZ and A. ROSENBERG, Arkiv Kemi *8* (1956), 23.
7. S. L. MATLOW and G. W. WHELAND, J. Am. Chem. Soc. *77* (1955), 3653.
8. C. K. INGOLD, Structure and Mechanism in Organic Chemistry. London 1953, p. 262, Table 20-4.
9. B. PULLMAN and A. PULLMAN, Les théories électroniques de la chimie organique. Paris 1952, Chap. X.
10. R. D. BROWN, Quart. Revs. *VI* (1952), 63.
11. L. MELANDER, Arkiv Kemi *2* (1950), 211.
12. K. HALVARSON and L. MELANDER, Arkiv Kemi *11* (1957), 77.
13. P. B. D. DE LA MARE, T. M. DUNN, and J. T. HARVEY, J. Chem. Soc. (London) *1957*, 923.
14. C. REID, J. Am. Chem. Soc. *76* (1954), 3264.
15. N. MULLER, L. W. PICKETT, and R. S. MULLIKEN, J. Am. Chem. Soc. *76* (1954), 4770.
16. G. OLÁH *et al.*, Nature *178* (1956), 693, 1344; *179* (1957), 146.
17. S. SUNNER, Acta Chem. Scand. *9* (1955), 847.
18. B. H. CHIRGWIN and C. A. COULSON, Proc. Roy. Soc. *A 201* (1950), 196.
19. O. CHALVET and R. DAUDEL, Compt. rend. *241* (1955), 413.
20. R. DAUDEL and O. CHALVET, J. chim. phys. *53* (1956), 943.
21. H. D. HARTOUGH, Thiophene and Its Derivatives. New York 1952.
22. B. M. WEPSTER, Rec. Trav. Chim. P.-B. *75* (1956), 1473.
23. C. D. HURD and K. L. KREUZ, J. Am. Chem. Soc. *74* (1952), 2965.
24. C. A. COULSON and J. DE HEER, J. Chem. Soc. (London) *1952*, 483.
25. C. A. COULSON and H. C. LONGUET-HIGGINS, Proc. Roy. Soc. *A 193* (1948), 456.

Tryckt den 26 juni 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 43

Vorgelegt am 10. April 1957

Zur Kinetik der Reduktone und Pseudo-Reduktone. II

Von Hans von Euler und Hans Hasselquist

Mit 6 Figuren im Text

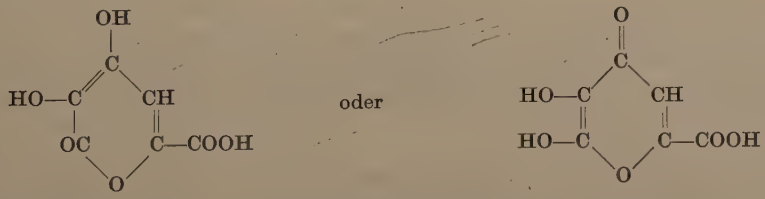
Das Tillmans-Reagens (TR. Dichlorphenol-indophenol) wird bekanntlich als charakteristische Test-Substanz für Reduktone verwendet, seit Tillmans (1), Zilva (2) und Szent-Györgyi (3) dasselbe zum Nachweis von Ascorbinsäure einführten. Im Verlauf von 25 Jahren hat sich gezeigt, dass TR sich auch zur *quantitativen* Bestimmung von aci-reduktonen (4) und Reduktonaten gut eignet.

Zur Kennzeichnung verschiedener Klassen von Reduktonen begannen Euler, Hasselquist und Ceder (5) eine Untersuchung über den *zeitlichen Verlauf* der TR-Reduktion *bei variierendem pH*. Solche Messungen führen zu einer erweiterten und vertieften Einsicht in die Chemie der Reduktone und werden in unserem Arbeitskreis systematisch fortgesetzt.

Bei den untersuchten aci-Reduktonen zeigen die Kurven, welche die Zeiten der TR-Reduktion mit pH in Beziehung setzen, ein Maximum (bei diesem ist das Produkt: Konzentration × Aktivität der reagierenden Molekülart ein Minimum).

Der Einfluss eines Puffers auf die Lage des erwähnten Maximums sowie auf die absolute Grösse der Reaktionszeiten beruht z.T. darauf, dass das Puffersalz sich mit dem Alkali bezw. dem Redukton-TR-Salz umsetzt. Unsere neuen Messungen an Ascorbinsäure (6) zeigen in Bestätigung unserer früheren Beobachtungen (5) dass das Maximum durch 1/15 mol. Phosphatpufferlösung um 2 pH-Einheiten (pH 9 – 11) verschoben wird. *Eine analoge* Wirkung zeigte sich im System Ascorbins., O_2, Methylenblau (7).

Oxykomensäure. Einen gleichartigen pH-Einfluss auf das Maximum der Reduktionszeiten wie bei den Versuchen mit Ascorbinsäure findet man bei den Versuchen mit Oxykomensäure.



Ein Vergleich der Kurven in Figuren 1a und 1b zeigt die wesentliche Verminderung der Reaktionszeiten (116 Sekunden gegen 18 Minuten); also eine Erhöhung der Reaktionsgeschwindigkeit, die der Puffer durch einen Einfluss auf pH erzeugt.

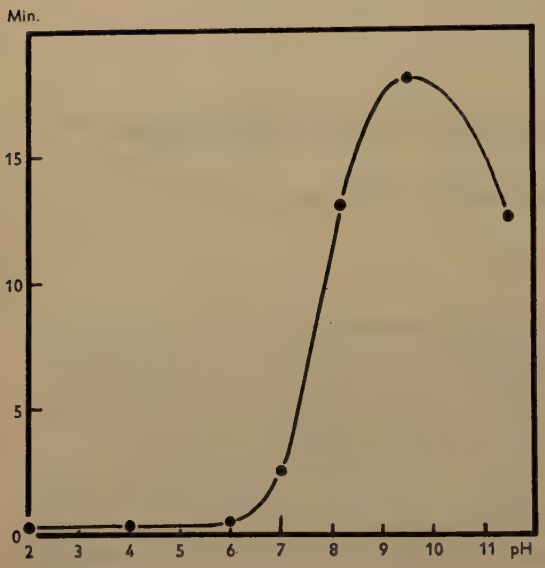


Fig. 1 a. Oxykomensäure ohne Puffer.
OKS 0,005 mol. 1 ml
TR 0,005 mol. 0,8 ml

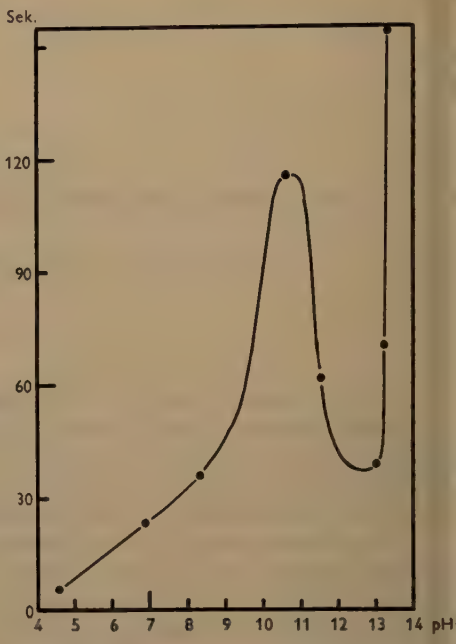


Fig. 1 b. Oxykomensäure mit Puffer.
OKS 0,005 mol. 1 ml
TR 0,005 mol. 0,8 ml
PO_4-Puffer $^1/_{15}$ mol. 0,2 ml

Das gleiche Resultat ergibt der folgende Versuch, bei dem die TR-Konzentration viel kleiner war (0,1 statt 0,8).

2 ml 0,02 mol. OKS
2 ml Natronlauge } pH 10,55 Entfärbungszeit in Sek. 11,8

2 ml 0,02 mol OKS
2 ml 1/15 m. PO_4 Puffer + NaOH } pH 10,92 » in Sek. 10,9

Zu jeder Probe 0,4 ml 0,01 n. TR.
pH-Konstanz während des Versuchs kontrolliert.

Man bemerkt in Fig 1b den durch die Zunahme der Mono-anionen der Oxykomensäure bedingten starken Abfall der Reaktionszeiten zwischen pH 10,5 und 12. Der Einfluss von Alkali auf die Oxykomensäure besteht zunächst in einer 2-stufigen Neutralisation, sodann in einer Sprengung des Pyronringes (Fig. 2).

Von pH 12,5 an werden unter der Einwirkung der hohen Alkali-Konzentrationen die reaktionsvermittelnden Ionen zerstört, wodurch die Reaktionszeit wieder steil ansteigt.

Gegen Jod verhält sich die Oxykomensäure ganz analog wie Ascorbinsäure.

Reduktone, deren Endiolgruppe nicht mit einem Carbonyl (ev. einem anderen sauren Rest) benachbart ist, wie z. B. Benzoin oder Dioxyaceton; sind nur als *Reduktonate* wirksam, u. zw. zunächst durch die Reduktonat-mono-anionen. Die Reaktionszeiten nehmen von etwa pH 7 an mit steigendem pH zu.

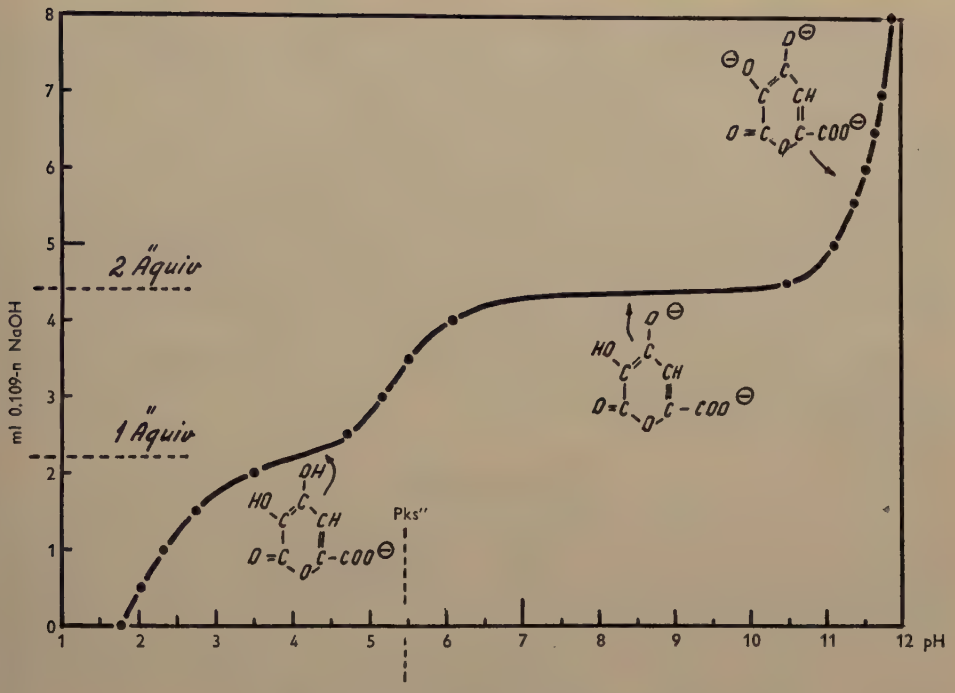


Fig. 2. 2,5 ml 0,1 mol. Oxykomensäure.

Als Beispiel für ein aromatisches Reduktonat dient *Pyrocatechol (Brenzcatechin)*, dessen Diss. Konst. $K_1 = 3.10^{-10}$ gefunden wurde und dessen Aktivität bei veriierendem pH in Fig. 3 dargestellt ist.

Bei Alkalinitäten höher als pH 9,5 machen sich neben den Monoanionen auch die Di-anionen in steigendem Maass geltend.

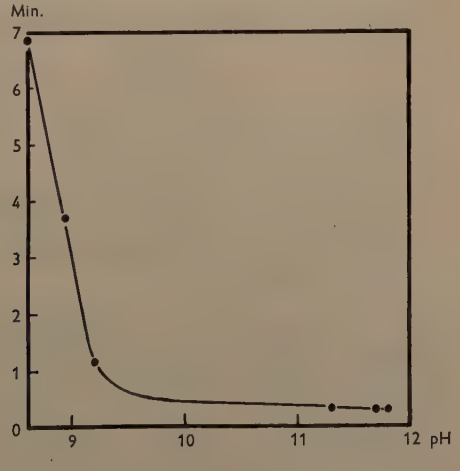


Fig. 3. Brenzcatechin.
Brenzcatechin 0,006 mol. TR 0,0005 mol.

Im oxydo-reduktiven Stoffwechsel spielen bekanntlich die Diphenole eine wichtige Rolle; die Oxydation der Ascorbinsäure durch Luftsauerstoff wird, wie schon SZENT-GYÖRGYI fand, durch Diphenole, u.z.w. nur im alkalischen Gebiet, gehemmt. Der Übergang der Ascorbinsäure in Dehydroascorbinsäure wird durch Spuren von Cu katalysiert; in diesem System üben *o*-Diphenole eine deutliche Schutzwirkung auf Ascorbinsäure und auf andere Wirkstoffe, z.B. Cozymase aus (8), und die Brenzcatechin-Derivate Adrenalin und Noradrenalin fungieren im oxydativen Stoffwechsel (9) unter Vermittlung ihrer Chinone als Katalysatoren.

Das Ascorbinsäure-System tritt gerade da hervor, wo — wie z.B. in den Augenlinsen — der Brenztraubensäure-Cyclus zurücktritt. (Euler, Naturwiss. Rundschau 1956, 83.) Auch Dihydroxy-Naphtaline sind in biochemischer Hinsicht bemerkenswert.

Werden die Enolformen der typischen Reduktonate angesäuert, so entstehen ihre Ketoformen, die bis etwa pH 7 gegen TR stabil sind.

Unter den Bezeichnung

Pseudo-Reduktone

haben wir eine grosse Klasse von Stoffen zusammengefasst (vgl. Mitt. I), welche *ähnlich wie Reduktone mit TR reagieren ohne die Endiolgruppe zu enthalten.* Zu den Pseudo-Reduktonen gehört u.a. eine Reihe von *Diketonen;* zu einer systematischen Untergruppierung dieser Stoffe reicht unser experimentelles Material noch nicht aus. Es sei auch betont, dass die Reduktionsfähigkeit mancher Diketone auch auf Endiolformen, z.T. unter Annahme von Chelatbindungen, zurückgeführt werden könnte. Für Stoffe vom Typus A könnte eine Valenzverteteilung nach dem Schema B in Betracht gezogen werden.

Zu untersuchen bleiben bei den Keto Enol-Systemen besonders [1]

1) die VAN'T HOFF-DIMROTHsche Regel:

$$\frac{\text{Enolform}}{\text{Ketoform}} = \frac{\text{Löslichkeit der Enolform}}{\text{Löslichkeit der Ketoform}}$$

(siehe hierzu auch (O. DIMROTH, Lieb. Ann. 377, 127 (1910) und W. DIECKMANN, Ber. 55, 2470; 1922),

2) die Beziehungen zwischen Enolisierungs-Tendenz, elektrochemischem Charakter und Energie der Komponenten des Elektronen-Systems.
3) Mechanismus und Geschwindigkeit der Keto-Enol-Umlagerungen und ihren Temperatur-Koeffizienten.[1]

Die künftige Kinetik der Keto-Enol-Systeme wird nicht nur die einzelne gerichteten Valenzen (Valenzwinkel und Bindungslängen), sondern auch die Feldstärken der reagierenden Ionen und das Kraftfeld des gesamten Moleküls zu betrachten haben.

[1] Siehe hierzu auch Henecka, Chemie der β-Dicarbonyl-Verbindungen (Springer-Verlag, 1953).

Eine Reihe von Diketonen (vgl. Mitt. I.) reduziert TR *weder* in saurer *noch* in alkalischer Lösung.

TR reduzierende Diketone

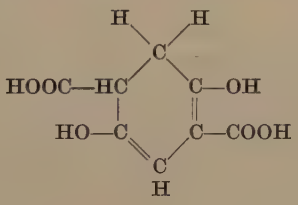
α) Reduktion in alkalischer Lösung

Diacet-bernsteinsäureester (10). Wie schon in der Mitt. I erwähnt, reduziert dieses 1,4-Diketon TR in langsamer Reaktion zu 85%.

Trotz der geringen Löslichkeit wurde der Diacet-bernsteinsäureester zu Injektionen in Yoshida-Ascites-Tumor-Ratten verwendet. Durch 5 aufeinander folgende intraperitoneale Tagesdosen von 5 mg nach intraperitonealer Ascites-Injektion wurde die Lebensdauer der Versuchsratten um etwa 5 Tage verlängert.

Succinylo-bernsteinsäureester, aus Bernsteinsäure-diäthylester, Äthylacetat und Natrium nach H. LIEBERMANN (11) dargestellt und in Form gelbgrünlicher Blättchen vom Schmpt. 126° erhalten.

1 Mol. des Succinylo-bernsteinsäureesters verbraucht in alkalischer Lösung 0,97 Mol. TR, dagegen tritt weder in neutraler noch in saurer Lösung eine TR-Reduktion ein. Ausser der sich von der Diketoform ableitenden Dienolform kommt noch die Form C in Betracht.



C

β) Reduktion in saurer Lösung

α,α'-Diketo-adipinsäure-ester, dargestellt nach KUHN und DURY (12). Den Äthylester erhielten wir aus Äthylacetat krystallisiert in länglichen spitzigen Krystallen vom Schmpt. 160°.

Reduziert TR in 50-%igem Äthanol per Mol. zu 80 %. Nach Zusatz von Alkali tritt kein weiterer Verbrauch an TR ein.

Titration mit Jod. Per Mol. Diketo-adipinsäureester wurden 1,04 Mole J_2 verbraucht.

6,6 mg Diketo-adipinsäureester, in Äthanol gelöst, mit Wasser auf das gleiche Volumen verdünnt und mit Alkali titriert (Bromthymolblau als Indikator) ergab mit NaOH einen Umsatz von 18%. In einem weiteren Versuch ergab sich der Wert von $pK_S = 6$.

Nach WILLE (13) reduziert der Dimethylester der α,α'-Diketo-adipinsäure 88 % des TR.

Benzoyl-formoin, $C_6H_5 \cdot CO \cdot \underset{OH}{CH} \cdot CO \cdot CO \cdot C_6H_5$, dargestellt nach ABENIUS u. SÖDERBAUM (14) aus Phenylglyoxal + KCN in 50% igem Äthanol. Schmpt. unseres

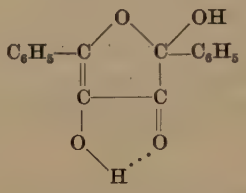
Präparates, gelbe, harte Prismen, aus Äthanol 189,5°. Dreimal aus Methanol umkrystallisiert entstanden gelbgrüne Stäbchen, die bei 189,5° konstant schmolzen.

Die TR-Titration in saurer Lösung (unter Bildung des Tetraketones) ergab

E_{gef} 120 $E_{ber\ f.\ C_{16}H_{12}O_4}$ 134

Jodverbrauch, gemessen von KARRER u. Mitarb. (15).

Eine Halbacetal-Formel (Chelatbindung) schlägt KARRER vor (16).



Bei Zusatz von $FeCl_3$ in Methanol-Äthanol-Lös. verläuft die Reduktion zu $FeCl_2$ so schnell, dass die Färbung des Endiol-Komplexes kaum wahrnehmbar ist.

In konz. Schwefelsäure färbt sich das Benzoylformoin gelb, in der Wärme schwarzbraun. Beim Schmelzen des Formoins tritt Rotfärbung auf (17), bei 240° schwache Gasentwicklung.

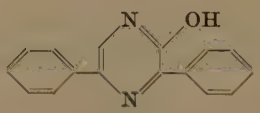
Versetz man die Benzoylformoin-Lösung unter Stickstoff mit 12%iger Ammoniaklösung, so bildet sich sofort eir stark gelbrotes Ammoniumsalz. Die beim Erhitzen auf dem Wasserbad ausfallende Masse krystallisiert beim Abkühlen und liefert grosse, gelbe, grünlich fluorescierende Nadeln vom Schmpt 272–273°. Dieselbe reduzieren TR nicht.

Analyse	Ber. für $C_{16}H_{12}ON_2$(248,3)	C 77,40	H 4,87	N 11,29
	Gef.	77,16	4,56	11,50

Wir nehmen an, dass dieses Produkt nach folgender Formel gebildet wird:

$$C_{16}H_{12}O_4 + 2NH_3 - 3H_2O \rightarrow C_{16}H_{12}ON_2$$

Die Identität mit 2-oxy-3,5-diphenylpyrazin steht nunmehr fest. Wir nehmen also die folgende Formel an:



An Ratten, die gleichzeitig mit intraperitonealer Injektion von Yoshida-Ascites Tumor 4 Tagesdosen von je 3 mg Benzoylformoin intraperitoneal erhielten, wurde eine wesentliche Verlängerung der Lebensdauer von 6 auf 15 Tage festgestellt. Ausserdem scheint das injizierte Benzoylformoin die sonst oft nach Ascites-Injektion im Peritoneum auftretenden Blutungen zu hemmen.

Zur weiteren Kennzeichnung des (gegenüber TR und J_2) untersuchten Pseudo-Reduktone geben wir hier das Verhalten ihrer Lösungen in 50%igem Äthanol auf Zusatz von alkoholischer $FeCl_3$-Lösung an.

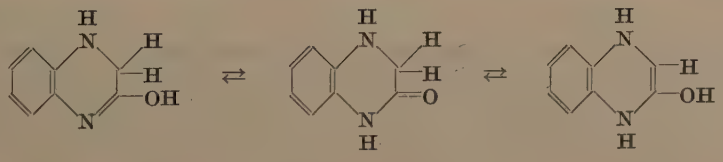
Verhalten zu TR	Farbenreaktion
In saurer Lösung mit TR reagierend	
α,α′-Diketo-adipinsäure	rasch vorübergehend rotviolett
Benzoylformoin	rasch vorübergehend schwarzviolett
In alkalischer Lösung TR reduzierend	
Diacet-bernsteinsäureester	keine Farbe
Succinyl-bernsteinsäureester	rotviolett
Hexandion-1,2	schmutzigbraunrot
TR nicht reduzierend	
Acetylaceton	schwach braunrot
Acetonylaceton	keine Farbe
Phenylacetyl-acetophenon	braunrot
Oxalyl-diacetophenon	braunrot
Oxal-diessigsäureester	braunrot
2-Benzoyl-cyclopentanon	intensiv kirschrot
1,2,3-Tribenzoyl-cyclopropan	keine Farbe
Dimedon (5,5-Dimethyl-cyclohexandion)	keine Farbe

Es handelt sich hier um die Bildung von Ketol-Fe^{III}-Komplexen und ev. um die Reduktion von Fe^{III} zu Fe^{II}. Auf die elektrometrische und spektrometrische Charakterisierung der *Ketole* kommen wir in einer folgenden Mitteilung zurück.

Im Anschluss an frühere Versuche mit 3,4-Dihydroxy-furan-dicarbonsäureester haben wir untersucht, wie die TR-Reduktion dieses Pseudo-Reduktons von pH abhängt. Die in Fig. 4 angegebenen Reaktionszeiten sind wesentlich höher als die für die aci-Reduktone Triose-Redukton und Ascorbinsäure gefundenen. Die Reaktionszeiten sind bestimmt durch die Geschwindigkeiten der Spaltung des Furanringes bezw. durch die Geschwindigkeit, mit welcher der Dihydroxy-furansäureester in die Dienolform übergeht.

Unter den verschiedenen Klassen von Pseudo-Reduktonen zugehörenden Substanzen sei hier auch das *Dihydro-oxy-chinoxalin* erwähnt.

Wir haben es aus *o*-Phenylen-diamin + Monochloressigsäure durch Behandeln dieser Mischung mit Zinkpulver nach MOTYLEWSKI (Ber. 41, 800; 1908) dargestellt. Die reine Substanz wurde in Form kleiner schwach gefärbter Nadeln erhalten, die nach dem Trocknen bei 132–133° schmolzen (mit 1 Mol Krystallwasser Schpt. 96–97°).



In wässriger Lösung reduzierten 2,2 mg die Sbst. 0,01-n TR *nicht*. Nach Zusatz von 0,1 ml 2-n. NaOH trat ein langsamer TR-Verbrauch ein, und zwar reduzierten indiesen alkalischen Lösung 1 Mol Dihydro-oxy-chinoxalin 0,84 Mole TR.

Bezüglich der Proton-Wanderung bei solchen Übergängen sei auf eine bemerkenswerte Arbeit von G. BRIEGLEB u. W. STROHMEIER (Angew. Chem. 64, 409; 1952) verwiesen.

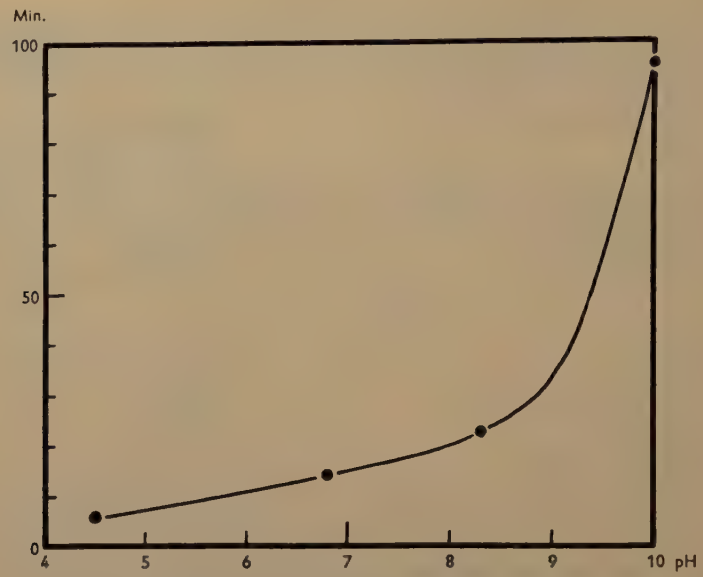


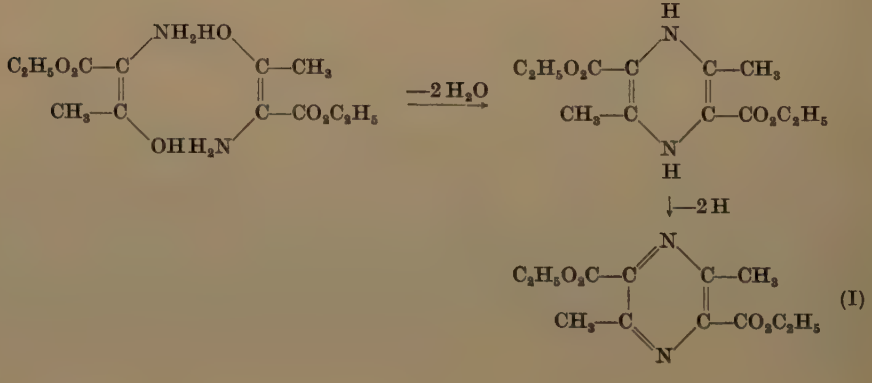
Fig. 4. 3,4-Dihydroxy-furan-dicarbonsäuremethylester.

Ester	0,005 mol.	1 ml
TR	0,01 mol.	0,8 ml
PO_4-Puffer	$^1/_{15}$ mol.	0,2 ml

Reduktionswirkungen von Enaminolen und Endiaminen bei variierender Acidität

Enaminole und Endiamine verhalten sich gegen TR zum Teil wie ächte Reduktone, une wurden zunächst als solche angesehen. Erst eine spätere genaue Prüfung hat Unterschiede bezüglich ihres reduktiven Verhaltens erkennen lassen.

Offene Enaminole. Den von MARTIUS und KNOOP (18) studierten α-Amino-β-keto-buttersäureester haben wir nach S. GABRIEL und TH. POSNER (19) dargestellt. Wir titrierten direkt 8,8 mg des kryst. Hydrochlorides in 10 ml Wasser mit 0,01 n. TR-Lösung und fanden E = 187, während sich E für $C_6H_{12}O_3NCl$ zu 91



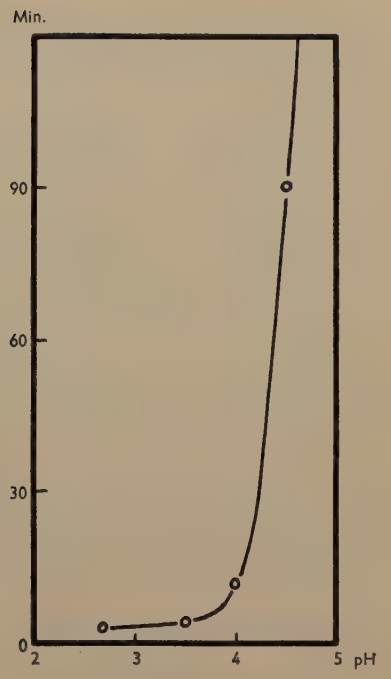


Fig. 5. *o*-Phenylendiamin.

o-Phenylendiamin	1 ml	0,005 mol.
TR	0,8 ml	0,005 mol.
PO_4-Pufferlös.	0,2 ml	$^1/_{15}$ mol.

berechnet. Das Enaminol zeigte also *nur die Hälfte der erwartenden Reduktion.* Man kann diesen Befund darauf zurückführen, dass sich aus 2 Molekülen des Enaminols zum Teil zunächst intermediär 1 Mol Dihydro-pyrazin(J) bildet[1], das sich mit TR umsetzt.

Cyclische Enaminole. Wir erwähnen, wie schon früher (Monogr. S. die Aminotetronsäure von Wolff u. Lüttringshaus (20) bezw. von Micheel u. Hasse (21), sowie die Scorbaminsäure von Micheel u. Mittag (22). Ferner das Uramil sowie das 3-Amino-4-hydroxy-cumarin von Arndt u. Mitarb. (23), schliesslich die Tetronimide Dahn u. Mitarb. (24).

Als *aromatisches Enaminol* ist auch das *o-Aminophenol* anzuschliessen.

Endiamine (vgl. dieses Arkiv 11, nr 1; 1957).

Die neuerdings von Brederecк (25) bearbeitete *tetramere Blausäure,* $(HCN)_4$, welcher von manchen Forschern die Struktur eines offenen Endiamins zugeschrieben wurde, während Hinkel (26) eine Amino-Imino-Formulierung bevorzugt, reduziert das TR in neutraler und schwach saurer Lösung. (pH ~ 4).

Jod-Verbrauch, 2,6 mg $(HCN)_4$ verbrauchten 0,0335 Äquiv. J_2, also 70% des für die Endiamin-Struktur berechneten Wertes, entsprechend dem mit TR erhaltenen Ergebnis. Wie

[1] Lässt man die wässrige Lösung des Amino-keto-buttersäureesters mit etwas KAc an der Luft stehen, so krystallisiert nach kurzer Zeit Di-methyl-pyrazin-dicarbonsäureester vom Schmpt. 86° aus. Durch diese Kondensation sowie durch die Gegenwart von NH_4Cl wird die Reinigung des Amino-keto-buttersäureesters erschwert.

viel davon dem als Endiamin vorhandenen Anteil unseres Tetrablausäure-Präparates zukommt, können wir noch nicht entscheiden. Einstweilen zählen wir die tetramere Blausäure den Pseudo-Reduktonen zu.

Unter den von BREDERECK studierten Tetrablausäure-Derivaten sind besonders die mit α-Dicarbonyl-Verbindungen, mit Imidoestern und mit Orthoestern erhaltenen zu nennen.

Aromatische Endiamine. Das *o*-Phenylendiamin (Diss. Konst. $K_{25} = 3.10^{-10}$) reduziert TR in recht stark saurer Lösung (pH ~ 4) in der es zum grossen Teil in Kationen-Form vorliegt. Die Reaktionszeit mit TR nimmt mit steigendem pH, also mit steigender Salzbildung, zu, wie Fig. 5 zeigt.

1 Mol *o*-Phenylendiamin verbraucht bei pH ~ 6 2,1 Mole J_2. Wird *o*-Phenylendiamin bei variierendem pH mit Jod (Stärke als Indikator) titriert, so zeigt sich, dass die Reaktionszeit mit zunehmendem pH abnimmt. Der pH-Einfluss ist also dabei entgegengesetzt dem für die TR-Reduktion festgestellten (Fig. 5).

Heterocyclische Endiamine. Diamino-Pyrimidine sind schon zeitig zu Synthesen verwendet worden (vgl. EULER u. HASSELQUIST, Monographie s. 19). Aus 3,4-Diamino-uracil (BREDERECK (27) können Pteridine (28) gewonnen werden. Unter den interessanten Produkten solcher Synthesen sind die *Pteroyl-glutaminsäure* sowie das Vitamin *Folsäure* hervorzuheben.

Herrn Ingenieur H. WÅHLSTAM danken wir für die Mitarbeit bei den Versuchen.

Stockholm, Universität, Institut für organ. Chemische Forschung.

LITERATUR

1. TILLMANS, J. und Mitarb., Z. Unters. d. Lebensmittel, 63, 3 (1932).
2. ZILVA, S. S., Biochem. J. 18, 186 (1923) u. 21, 689 (1927).
3. SZENT-GYÖRGYI, A. v., Biochem. J. 22, 1387 (1928).
4. Zur Nomenklatur der Reduktone siehe EULER, Kem. Arb. N. F. B, 16, III (1954).
5. EULER, HASSELQUIST H. und CEDER, O., Lieb. Ann. Chem. 581, 198 (1953). — Siehe auch Arkiv Kemi 6, nr 44 (1953).
6. EULER und HASSELQUIST, Arkiv Kemi 11, Nr 1 (1957).
7. EULER, MYRBÄCK, K. und LARSSON, H., Z. physiol. Chem. 217, 8 (1933).
8. EULER, und HASSELQUIST H., Arkiv Kemi 8, nr 21 (1955).
9. GREEN, D. B. und RICHTER, D., Biochem. J. 31, 596 (1937).
10. WILLSTÄTTER, R. und CLARKE, C., Ber. deutsch. chem. Ges. 47, 293 (1914). — KNORR, L. und KAUFMANN, H. P., Ber. deutsch. chem. Ges. 55, 232 (1922). — KAUFMANN, H. P., ebenda 56, 2523 (1923).
11. WISLICENUS, W., Ber. deutsch. chem. Ges. 20, 590 (1887). — LIEBERMANN, H., Lieb. Ann. Chem. 404, 287 (1914).
12. KUHN, R. und DURY, K., Lieb. Ann. Chem. 564, 34 (1949).
13. WILLE, Lieb. Ann. Chem. 538, 249 (1939).
14. ABENIUS, R. V. und SÖDERBAUM, H. G., Ber. deutsch. chem. Ges. 24, 1381 (1891).
15. KARRER, P. und Mitarb., Helv. Chim. Acta 18, 273 und 1145 (1935).
16. Siehe auch Houben-Weyl, Methoden d. organ. Chem. 4. Aufl. Bd II.
17. BLATT, A. H., J. Am. Chem. Soc. 58, 1897 (1936).
18. MARTIUS, C. und KNOOP, F., Z. physiol., Chem. 240, 195 (1936).
19. GABRIEL, S. und POSNER, TH., Ber. deutsch. Chem. Ges. 27, 1142 (1894).
20. WOLFF, L. und LÜTTRINGHAUS, A., Lieb. Ann. Chem. 312, 158 (1900).
21. MICHEEL, F. und HASSE, Ber. deutsch. Chem. Ges. 69, 879 (1936).

22. MICHEEL, F. und MITTAG, R., Z. physiol. Chem. 247, 34 (1937).
23. ARNDT, F. und EISTERT, B., Ber. deutsch. Chem. Ges. 68, 1572 (1935).
24. DAHN, H. und Mitarb., Helv. 37, 1309 u. 1612 (1954). — DAHN, H., und HAUTH, H., Helv. 39, 1366 (1956).
25. BREDERECK, H. und Mitarb., Lieb. Ann. Chem. 600, 81 u. 86 (1956).
26. HINKEL, L. E. und Mitarb., J. Chem. Soc. 1937, 1432.
27. BREDERECK, H. und Mitarb., Chem. Ber. 86, 321 (1953).
28. PFLEIDERER, W., Angev. Chem. 68, 533 (1956).

Tryckt den 30 juli 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Bd 11 nr 44

Vorgelegt am 24. April 1957

Chemische und biologische Versuche mit Pseudo-Reduktonen und Derivaten derselben

Von Hans von Euler und Hans Hasselquist

Mit 1 Figur im Text

Wie vor kurzer Zeit mitgeteilt wurde (1) fanden wir für Benzoyl-formoin

$$C_6H_5–CO–\underset{\displaystyle OH}{\underset{|}{CH}}–CO–CO–C_6H_5$$

bei der Titration mit Tillmans-Reagens (TR) den E-Wert 120, während sich für $C_{16}H_{12}O_4$ der Wert 134 berechnete.

Das Benzoyl-formoin wurde durch ein Verfahren dargestellt, das sich dem von Abenius und Söderbaum (2) angegebenen anschliesst.

1 g frischdestilliertes Phenylglyoxal wurde unter Rühren mit einer Lösung von 100 mg KCN in 1 ml 50%igem Methanol versetzt. Das gelbgrüne Phenylglyoxal färbte sich unter Wärme-Entwicklung sofort rotbraun. In die nach dem Abkühlen zähe Masse wurde langsam Wasser eingerührt. Die dabei sich allmählich ausscheidenden Kristalle wurden abfiltriert, gewaschen und getrocknet und schmolzen bei 175–178°. Dieses in fast quantitativer Ausbeute entstehende Rohprodukt liefert nach Umkristallisieren aus wenig Äthanol gelbe Stäbchen, die unter Rotfärbung bei 189–189,5° schmelzen.

Einwirkung von Ammoniak auf Benzoylformoin

Darstellung der Sbst. 261

Das nach obigem Verfahren dargestellte Benzoyl-formoin reagiert auf Zusatz von 12%iger Ammoniaklösung unter Stickstoff sofort wie früher (1) beschrieben, unter Bildung eines gelbroten Ammoniumsalzes.

Beim Erhitzen auf dem siedenden Wasserbad ging das Salz unter Braunfärbung in Lösung. Aus dieser schied sich nach 10 Minuten ein Öl aus, nach 2 Stunden hatte sich das ganze Reaktionsprodukt als eine zum Teil kristallinische dunkelbraune Masse ausgeschieden. Die Lösung nahm dabei eine zitronengelbe Farbe an.

Nach Zusatz von HCl bis etwa pH 4 und Abkühlung erstarrte die Masse vollständig, wurde von der Lösung durch Dekantieren getrennt, mit Wasser gewaschen und getrocknet.

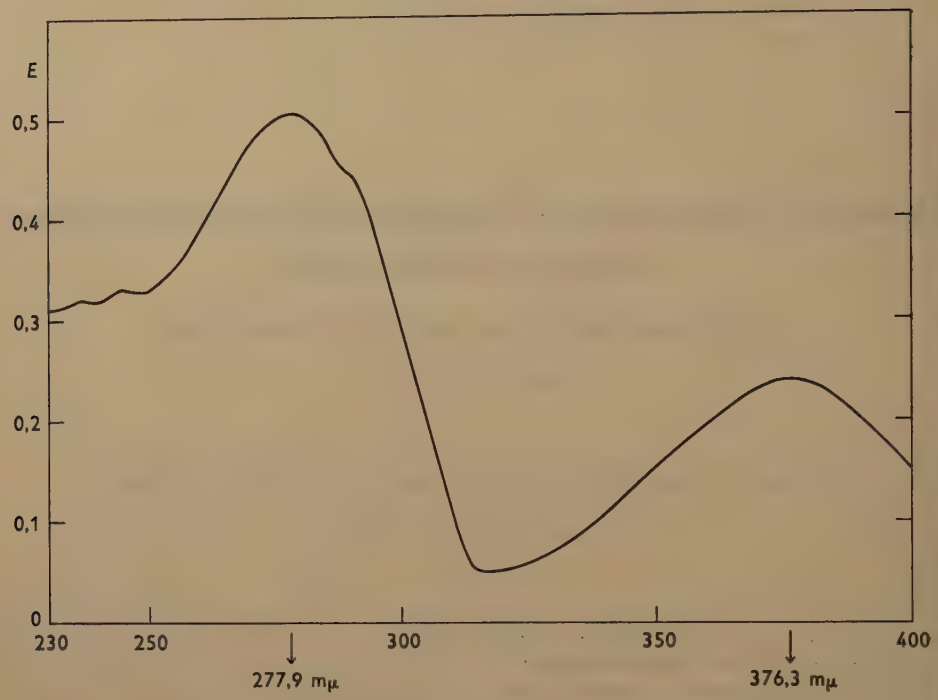


Fig. 1. 6,6 mg/100 ml Äthanol-Lösung. Substanz 261.

Die Kristallmasse wurde mit Methanol verrieben, wobei die schmierigen Produkte leicht in Lösung gingen. Ungelöst hinterblieb ein gelbes Kristallpulver, das abfiltriert und mit Methanol gewaschen wurde.

Ausbeute an Rohprodukt: 0,5 g vom Schmpt. 193–210°.

Diese Substanz wurde nun mit viel (ca. 75 ml) Aceton aufgekocht. Beim Stehen über Nacht in geschlossenem Gefäss schied sich eine Mischung ab von kleinen gelben und mikroskopisch kleinen grünen Nadeln.

Das Filtrat wurde nun bis zur beginnenden Kristallisation eingedunstet, wobei sich als Hauptprodukt grosse, gelbe grünlich schimmernde Nadeln vom Schmpt. 272° ausfielen. Durch weiteres Umkristallisieren aus Aceton konnten kleine Mengen einer intensiv grünen in Aceton schwerlöslichen Substanz abgetrennt werden.

Die vollständig gereinigten gelben Nadeln schmolzen bei 272–273°, wobei Sublimation eintritt.

Aus der früher angegebenen Analyse ergab sich die Zusammensetzung

$$C_{16}H_{12}ON_2 \ (248{,}3)$$

Die Sbst. 261 reduziert TR weder in saurer noch alkalischer Lösung. Sie löst sich in konz. Schwefelsäure mit intensiv gelbroter Farbe und kann daraus mit Wasser unverändert wieder ausgefällt werden. Die alkoholische oder azetonische Lösung fluoresziert kräftig in blau. Die Substanz ist ziemlich leichtlöslich in Aceton und Pyridin, schwerer in Methanol, Äthanol; unlöslich in Benzol, Petroleumäther und Wasser.

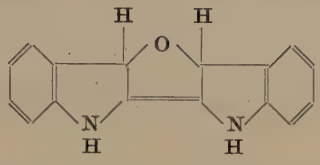
Wir nehmen an, dass sich die Substanz 261 nach folgender Gleichung bildet:

$$C_{16}H_{12}O_4 + 2\,NH_3 - 3\,H_2O \rightarrow C_{16}H_{12}ON_2$$

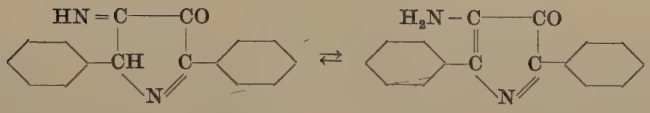
Das UV-Spektrum (HANS WÅHLSTAM) deutet auf ein N-haltiges Ringsystem hin (Fig. 1).

Wir ziehen deshalb folgende Formulierungen A oder B in Betracht:

A



B

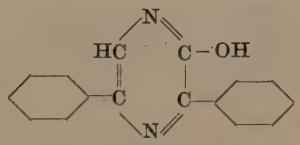


Indessen zeigt die vollständige Übereinstimmung der Sbst. 261 mit dem durch DUNN u. Mitarb. dargestellten 2-oxy-3,5-diphenyl-pyrazin, dass der Sbst. 261 die Formel C zukommt.

Äthanol-Lösung

DUNN u. Mitarb.	Maxima	2780 Å	$\varepsilon = 19300$
		3720 Å	$\varepsilon = 9200$
H. WÅHLSTAM	»	2779 Å	$\varepsilon = 19200$
		3763 Å	$\varepsilon = 9150$

C



Wurde Benzoyl-formoin in 50%iger Äthanol-Lösung mit äthanolischer $FeCl_3$-Lösung versetzt so trat eine schnell wieder verschwindende rotviolette Farbe auf, ganz ähnlich wie mit α,α'-diketo-adipinsäure-ester und Succinylo-bernsteinsäure-ester.

2-Benzoyl-cyclopentanon lässt dagegen mit $FeCl_3$ eine intensiv kirschenrote Farbe entstehen.

Biologische Wirkungen des Benzoyl-formoins

Intraperitoneale Injektion von 12 mg der in Wasser schwerlöslichen suspendierten Substanz (an 4 aufeinanderfolgenden Tagen je 3 mg) rief eine Verlängerung der Lebensdauer bei 200 g schweren Yoshida-Ascites-Ratten bis auf 12 Tage gegenüber 5 Tagen der Kontrollversuche hervor.

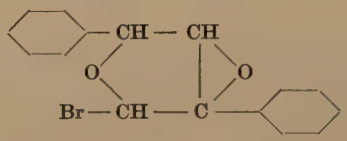
Gerstensamen, 8 bzw. 20 Stunden bei 20° eingeweicht in wässriger Suspension von 50 mg Sbst. in 100 ml Wasser (pH 5,0) wurden in ihrer Keimung um 2 bzw. 5 Tage verzögert. Keine Chlorophyllhemmung.

Brom-diphenacyl

Darstellung nach V. FRITZ, Ber. deutsch. chem. Ges. 28, 3032 (1895).

10 g Bromacetophenon in 50 ml abs. Alkohol gelöst wurde unter Kühlung mit einer kalten Lösung von 0,6 g Na in 12 ml Alkohol versetzt. Nach 3stündigem Stehen verschwand der Geruch nach Bromacetophenon. Während dieser Zeit war ein Teil des gebildeten Bromdiphenacylis auskristallisiert. Nach Verdünnung mit Wasser wurde wiederholt ausgeäthert. Aus der eingeengten Ätherlösung wurde das Bromdiphenacyl in Form schwach gelber Kristalle zusammen mit einem Öl gewonnen. Das Reinprodukt, kleine farblose Nadeln schmolz bei 161°.

Die Substanz deren wahrscheinliche Konstitution (4) durch die folgende Formel angegeben wird,



reduziert TR in alkalischer Lösung äusserst langsam, vermutlich nicht durch eine Dienol-Bildung sondern dadurch, dass Br zunächst durch OH ersetzt wird, worauf Enolisierung eintritt. Dagegen wird Jod nicht reduziert.

Stockholm, Universität, Vitamin-Institut.

LITERATUR

1. EULER u. HASSELQUIST, H., Dieses Arkiv *11*, nr 43 (1957).
2. ABENIUS, R. V. u. SÖDERBAUM, H. G., Ber. deutsch. chem. Ges. *24*, 1381 (1891).
3. G. DUNN, G. NEWBOLD, D. RAMSAY, F. SRING u. SWEENY, J. Chem. Soc. 1949, 2707.
4. O. WIDMAN, Lieb. Ann. Chem. 400, 86 (1913).

Tryckt den 30 juli 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 45

Read 10 April 1957

The uni- and multi-point binding of cationic sulphato-chromium complexes by collagen

By K. H. Gustavson

In an earlier paper (1), an approximate estimate of the proportion of the uni- to multi-point binding of electropositive chromium complexes by collagen from aqueous solutions of basic chromium chlorides was communicated. It was found that the main part of the irreversibly fixed polyvalent complexes were unipointly attached to collagen. Only about ten per cent of the fixed chromium complexes had reacted multi-pointly with collagen. The aim of introducing chromium salts into the collagen structure of the skin, in the important technical application of chrome tanning, is to stabilize the protein, and this requires multipoint binding of the chromium complex to adjacent trihelical units of the protein. These chrome-complex bridges are exceedingly stable and cross-link the collagen structure very effectively. The important point of the findings in the previous paper (1) was that only a minor part of the chromium complexes combining with collagen are fixed bipointly and hence could contribute to the cross-linking and stabilization of the protein lattice.

Although the reaction of basic chromium chlorides with collagen is mainly of theoretical interest, since the corresponding sulphates are of greater technical importance, the use of basic chlorides was preferred for the initial investigation, since the system is easier to handle theoretically. The chlorides have one decided advantage over the basic sulphates in the study of the interaction of cationic complexes with collagen, viz. the fact that the chlorides form positively charged chromium complexes exclusively in aqueous solution, while solutions of basic sulphates may contain the whole register of complexes. A decided drawback of the basic chlorides is, however, the low degree of complexing ability of the Cl-group. In that respect the basic sulphates are preferable, particularly in view of the method used, which is based on the different degree of protolytic stability of the anions associated with the "chrome-collagen compound" formed by the reaction. However, the complications inherent in the formation of non-cationic chromium complexes in solutions of basic chromium sulphates can by largely overcome by selecting suitable types of compounds and experimental conditions for the reaction unfavourable to the formation of the non-desirable complexes. The method employed in the present series of investigation was that described in a previous paper (1) which should be referred to for details. Only the main points and any major exceptions from the earlier procedure will be given in this paper.

The main reactions in the process of fixation of basic chromium sulphates by collagen of the skin are: (i) the attraction of the electropositive complexes of the polynuclear chromium sulphates to the carboxyl ions of collagen; an ionic reaction taking

place in a protolyzed system, generally at pH about 3. (ii) The penetration of the discharged carboxyl group into the chromium complex, which results in the formation of an exceedingly stable coordinate-covalent bond (2). The attachment of one chromium complex to at least two carboxyl groups of adjacent chains of the collagen is a requisite for the cross-linking and stabilization of the protein (2). Since the size of the binuclear complexes of chromium which form the main constituents of the solutions of basic sulphates used is of the order of a few Ångström units, the carboxyl groups must evidently approach very closely in order to be able to react with the same chromium complex. Hence, the accessibility of these active groups and the steric conditions in the collagen lattice, particularly as the to interhelical distances are expected to be of governing importance for the multipoint reaction between the chromium complex and the collagen (2).

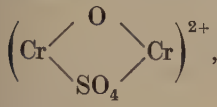
Experimental and results

Hide powder (Lyon) was the source of collagen. It was interacted in the hydrated state for seven days with the solution of the basic chromium sulphate in order to obtain practically complete inactivation of the carboxyl groups of the collagen. In the various series, a portion of 25 g of collagen was shaken with 500 ml of the solution of the chromium sulphate, containing 1 equiv. Cr per liter (25.3 g/l Cr_2O_3).

In additional series, the hide powder was treated, with continuous shaking, for three consecutive times with the solution of basic sulphate, applied at an initial concentration of one equivalent of Cr per liter each time. Each treatment lasted six hours. These series were included for the purpose of ascertaining whether any secondary chrome fixation by non-ionic valency forces takes place during the prolonged tanning period of seven days in the aforementioned series. Since the results from these series closely agreed with those of the first mentioned ones, it appears that any appreciable binding of chromium by groups other than the carboxyl ions of collagen did not occur.

Two types of basic chromium sulphates were used for the series forming the basis of the present paper, both containing practically all their chromium in the form of cationic complexes. These chromium compounds were analyzed by the conventional methods. The composition of the complexes, as to the number of sulphate groups complexly held (*sulphato* groups) was ascertained by the ion exchange technique (3) and the electrochemical behaviour of the chromium complexes by paper-ionophoresis (4). The first type of solution, designated as A, was obtained by reducing a 10% solution of sodium bichromate ($Na_2Cr_2O_7 \cdot 2\,H_2O$) with sulphur dioxide gas. The excess of SO_2 was removed by boiling the solution for 10 minutes, which also is necessary for the decomposition of any non-cationic chromium complexes present (5). The stock solution was diluted to a concentration of one equivalent of Cr per liter and aged two weeks before use, which time is sufficient to ensure the attainment of the protolytic equilibrium.

The composition of this solution corresponded to the empirical formula: $Cr_2(OH)_2(SO_4)_2 \cdot Na_2SO_4$, i.e., the 66.7% acid chromium sulphate. This solution contained 98% cationic chromium (ion exchanger: Dowex-50, in the sodium cycle). Using paper-ionophoresis all the chromium showed positive charge, although some heterogeneity was evident in the migration. The ion exchange analysis of the cationic complexes showed that the mean acidity of the sulphato-chromium complexes was 33%, corre-

sponding to one SO_4 group on two atoms of chromium. This type of chromium sulphate possesses on the average two atoms of chromium per complex (6). The cationic complexes may accordingly be represented by the schematic formula:  , which does not include the aquo groups coordinated on chromium (7).

The second type of basic chromium sulphate, designated as B, was a solution of a neutral-salt-free compound, its composition corresponding to the empirical formula: $Cr_2(OH)_2(SO_4)_2$. It was prepared by reducing an equimolar mixture of chromic acid and sulphuric acid by hydrogen peroxide (8). This solution was also employed at a concentration of one equivalent of Cr per liter. It contained cationic chromium complexed exclusively. The results of the various modes of characterization were identical with those given for the first solution, except for the figure of the sulphato-acidity which was 30% (9). Roughly, the same type of sulphato-chromium complexes are present in both solutions.

At the end of the interaction, when the final pH value of the residual solution was 3.0, the bulk of the solution in contact with the chromed hide powder was removed by filtering with suction. The chromed hide powder was then lightly washed in order to remove mechanically held chromium compounds. Since prolonged washing disturbs the original distribution of the sulphate groups in the chrome-collagen compound by the removal of SO_4 ions associated with the electropositive groups of collagen (10, 11), only a light washing with water was carried out.

The moist chromed hide powder, in portions corresponding to 2.0 g of collagen was equilibrated at pH 5 in one liter of water. The solution was continuously shaken and the acid diffusing out was continually neutralized by means of 0.1 *N* NaOH, as described in the previous paper (1). The reaction had gone to completion in 6–8 hours' time. By this treatment the acid compensating the basic protein groups is removed. Finally the chromed hide powder was analyzed for chromium, total bound sulphate and collagen by conventional methods.

The sulphate in the specimen neutralised at pH 5 is present in the form of (1) complexly bound SO_4 groups (*sulphato*) and (2) SO_4 groups electrostatically associated with the unipointly fixed sulphato-chromium complex. A check of the amount present is obtained by subtracting the number of mequiv. of acid removed per g collagen in the protolytic treatment at pH 5 from the corresponding amount present in the original chromed hide powder which was obtained by direct analysis.

The complexly bound sulphate was determined by analysis of the original chromed hide powder after it had been treated for 4 hours with continuous shaking, in portions corresponding to 2 g collagen, in 100 ml of a 4% solution of pyridine (11). At this pH (8.2), all ionically associated sulphate is removed. This method affords a rather sharp distinction between ionic and non-ionic sulphate groups. The pyridine-treated specimen contains all its sulphate in the form of sulphato-groups. By titration of the final pyridine solution (12), the amount of sulphuric acid extracted is estimated. Hence, a direct check is obtained of the amount of sulphate present in the electrovalent state in the chromed collagen, and also indirectly a check on the figure obtained by the direct analysis of the pyridine-treated specimen for its content of Cr and SO_4, on the basis of collagen.

The final results of the distribution of the three forms of sulphate groups, present

Table 1. Distribution of the sulphate groups in the chrome-collagen compounds.

In mequiv. per g collagen.

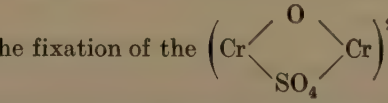
	Specimen from solution A	Specimen from solution B
Fixed chromium	5.18	5.20
Total bound sulphate	3.41	3.35
Complex-bound sulphate (Sulphato)	1.78	1.73
Sulphato groups + SO_4 groups electrostatically associated with the Cr complex	2.55	2.50
Protein-bound sulphate	0.86	0.85
SO_4 groups held electrostatically on the Cr complex	0.77	0.77
% of total Cr complexes unipointly fixed	89	89
% of total Cr complexes bipointly fixed	11	11

in the chrome-collagen compounds, are given in Table 1. The method by which the figures for the distribution of the anion were obtained is described in the previous paper (1).

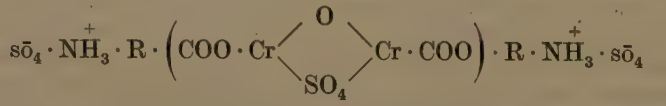
In the series in which the tanning was carried out by three consecutive operations as earlier mentioned, the specimen from solution A contained 5.32 mequiv. fixed chromium, and specimen B 5.48 mequiv. chromium. The figures of the distribution of the sulphate groups in the corresponding chrome-collagen compounds were of the same order of magnitude as those reported in Table 1.

Discussion

For an evaluation of the figures obtained, particularly the data on the proportion of uni-and bi-point fixation, an outline of the mode of deduction employed is needed.

Supposing the fixation of the  complex to have occurred solely

by means of two carboxyl ions of the collagen, the resulting chrome-collagen compound would be of the following general type:

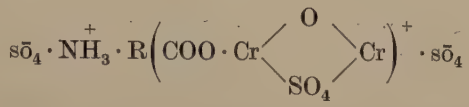


in which $\overset{+}{N}H_3 \cdot R \cdot \overset{-}{C}OO$ represents the protein, SO_4 the sulphate group, and $s\bar{o}_4$ one equivalent of the sulphate group.

The bound sulphate in this compound exists in *two* distinct states: (1) Complexly bound SO_4 groups in the chromium complex (*sulphato*). (2) Protein-bound sulphate,

the SO_4 ions being electrostatically balanced by the electropositive groups of the collagen, its NH_3^+ groups. This type of chrome-collagen compound will show the same ratio of SO_4 to Cr upon equilibration at any of the pH values used, pH 5 and 8.

The other extreme of reaction is that the bivalent sulphato-cromium cation reacts with one carboxyl ion of collagen exclusively, the unipoint fixation. The resulting chrome-collagen compound would then correspond to the following schematic formula:



In this instance, the bound sulphate exists in *three* different states of attachment (2). In addition to the two states mentioned in the foregoing example, a third form is present: the sulphate ions held by the chromium complex electrostatically (cf. ref. 10). While the complexly bound SO_4 groups, as well as the protein-bound sulphate, can be estimated by direct analysis, this is not the case with the third form. It is equal to the difference between the sum of ionically held sulphate, which is obtained by equilibration of the system at pH 8, and the amount of protein-bound sulphate which is removed on equilibrating the system at pH 5. This is less satisfactory than a direct estimate, particularly in view of the fact that the figure for the amount of this type of sulphate present forms the basis for ascertaining the extent of unipoint binding of chromium by collagen.

An additional source of complication is the possible redistribution of the sulphate ions, between the chromium complex and the basic protein groups (10, 11) on removing the protein-bound sulphate in the treatment at pH 5, the occurrence of which on prolonged treatment is indicated by the asymptotic shape of the curve of removed SO_4 against time. The difference in protolytic stability between these two forms of ionically associated sulphate is very much less than the difference in stability between last-mentioned forms and the sulphate present in the chromium complex (*sulphato*). Hence, only an *approximation* to the amount of the SO_4 ions held as counter-ions to the chromium complexes can be claimed.

However, it is possible to check the general trend of the results independently. This has been done and the result verifies the soundness of the idea and the method. The following considerations pertain to this verification. Since the acid-binding capacity of collagen is known to be 0.95 mequiv. as H_2SO_4 per g protein, in the form of the hide powder used, and moreover, since the total amount of sulphate in the original specimen of chromed collagen, as well as its content of sulphato groups, are known accurately, a combination of these figures will afford a control.

With specimen A, the content of ionically held SO_4 groups is 3.41–1.78 or 1.63 mequiv. SO_4 per g collagen. Further, since the acid-binding groups of collagen amount to 0.95 mequiv. per g collagen, it follows that at least 0.68 mequiv. SO_4 ions are present in the third form. Moreover, considering the presence of about 0.05 mequiv. of free acid-binding groups in the chromed hide powder, estimated by its interaction with 0.1 *N* solution of hydrochloric acid for one hour, correcting for the slight amount of acid consumed by the chromium complex on this brief interaction, an amount of 0.05 mequiv. SO_4 should be added to the minimum figure of 0.68 deduced, which gives the final value of 0.73 mequiv. SO_4 per g collagen held in electrostatic compensation of the chromium complex. The value obtained in this investigation is 0.77 mequiv. The fair agreement seems reassuring for the soundness of the method employed.

Further support for the concept advanced is given by the following consideration. A reaction involving the bipoint fixation of the binuclear chromium complexes exclusively would result in a maximum fixation of 6.8% Cr_2O_3, on the weight of collagen, whereas the amount found is 13.1% Cr_2O_3. This figure is close to the theoretical value of the maximum fixation of chromium on one-point fixation exclusively, which should be 13.6% Cr_2O_3.

Finally, it should be noted that the percentage of chromium complexes or carboxyl ions of collagen taking part in the reaction is arrived at in the following manner. In the chrome-collagen compound formed by unipoint fixation of the bivalent chromium complex exclusively, the number of equivalents of SO_4 groups electrostatically compensated by the single-charged binuclear chromium complex should be one sixth of the number of equivalents of chromium fixed by collagen. In the instance of series A, there should accordingly be $\frac{5.18}{6} = 0.86$ mequiv. SO_4 per g collagen in this form, if the reaction had run solely as a unipoint process. Since the number of these sulphate groups were estimated to be 0.77 mequiv. per g collagen, the percentage of the number of unipoint attached chromium complexes figured on the total number of chromium complexes bound is $\frac{0.77}{0.86} \times 100$, or 89%. Then indirectly, the multipoint reaction, probably mainly bipoint fixation, concerns only 11% of the participating chromium cations, or of the carboxylic ions of collagen involved in the fixation of chromium.

It is of interest to estimate approximately the amount of chromium fixed by collagen in terms of % Cr_2O_3, usually applied in stating the degree of chrome fixation. Then, in the present instance, the following considerations apply. The hide powder (collagen) used had a total amount of carboxyl groups of 1.26 mequiv. per g collagen (13) and contained 0.28 mequiv. amide groups (14). Hence, the free carboxyl groups will amount to about 1.0 mequiv. per g collagen. Allowing 0.1 mequiv. of the carboxyl groups for the binding of the proton, competing with the cationic chromium for the possession of the charged carboxyl groups at pH 3 of the system, as well as for any non-reacting carboxyl ions, an amount of 0.9 mequiv. of carboxyl ions would have reacted with the chromium complexes. The unipoint reaction would thus involve 0.81 mequiv. and the bipoint fixation 0.09 mequiv. carboxylic groups of the protein. Hence, the amount of fixed chromium, expressed as % Cr_2O_3 on the basis of collagen, would be as unipointly fixed: $0.81 \times 15.2 = 12.3\%$ Cr_2O_3; and as bipointly fixed: $0.09 \times 7.6 = 0.7\%$ Cr_2O_3, or in all 13.0% Cr_2O_3, all the figures being based on the weight of collagen. The figure of 5.18 mequiv. chromium in combination with the collagen in specimen A corresponds to 13.1% Cr_2O_3.

The amount of chromium incorporated into the collagen structure by the unipoint fixation would then be 95% of the total amount of chromium taken up and fixed by the protein. Thus, only a minor amount, or 5% of the total amount of fixed chromium, or 0.7% Cr_2O_3 on the basis of collagen, should be available for the cross-linking of the protein chains, resulting in stabilization of the structure, the main goal of the chrome tanning process. It should be noted that the present deductions are based on the supposition that the reaction is exclusively concerned with the carboxyl ions of the protein.

Since generally chrome leather with 3–4% Cr_2O_3 fixed by collagen resists the action of boiling water and proteinases, it is evident that the optimal cross-linking has been obtained. In the present series the maximum degree of stability of the collagen was attained upon short duration of tannage, in 2–4 hours, at this low level of fixed chromium. Hence, the bipoint fixation appears to be favoured in the early stage of

reaction, while the unipoint reaction is dominant in the late stage. A likely explanation is that the carboxyl ions on the exterior of adjacent tri-helical units are more easily accessible to the diffusing chromium complexes than the charged carboxyls in the interior of the unit, with its narrow interchain space.

Apart from broadening our concept of the nature of the chrome tanning process, the knowledge of the relative importance of the uni- and multi-point fixation of the chromium complexes by the charged carboxyl groups of collagen may be useful for the localization of adjacent carboxyl groups in the collagen lattice and also for the differentiation of the carboxyls in the interior and on the exterior of the tri-helical unit of collagen.

By an entirely different method (15), it has been indicated that the stabilizing effect of formaldehyde on collagen, which reaction involves the ε-amino groups of the lysine residues, is restricted to the cross-linking of a small fraction of these groups (about one out of every eight amino group) which must approach very closely for establishing the bridging by the methylene group. From corresponding studies of the stress-strain relationship as function of the number of cross-links of chromed tendon (collagen), findings similar to these reported in the present paper have been obtained (16). The results from interaction and cross-linking of collagen by fluoro-nitro-sulphones point in the same direction (17). Finally, from changes of the thermodynamic constants, imparted to the collagen by its fixation of basic chromium salts, Weir (18) concludes that the cross-linking of the protein units by the introduced chromium complexes attains its maximum on fixation of such a small amount of chromium as 1% Cr_2O_3; a figure very close to that obtained in this investigation.

SUMMARY

The mode of reaction of solutions of basic chromium sulphate with collagen (hide powder) has been investigated. An approximation to the proportion of *uni-* to *multi(bi)*-point fixation of cationic sulphato-chromium complexes by collagen on its equilibration with solutions of 66.7 % acid chromium sulphate, $Cr_2(OH)_2(SO_4)_2$, has been attempted.

The method is based on ascertaining the distribution of the sulphate groups present in the chrome-collagen compounds formed. The sulphate groups exist in three forms: (i) as sulphato-groups in the chromium complex; (ii) as protein-bound sulphate; and (iii) as counter-ion to the charged chromium complex unipointly attached to collagen. The rates of protolysis of the three forms differ. They have been estimated by equilibrating the systems at pH 5 and 8.

From the amount of electrostatically held sulphate, the percentage of unipointly fixed chromium complexes on the total number of reacting complexes can be approximated.

The unipoint reaction is dominant, accounting for about 90 % of the number of carboxyl groups of collagen taking part in the chrome fixation. Only about 10 % of these groups are able to combine with the bivalent sulphato-chromium-cation by complete discharge of the complex, i.e., bipointly. The latter type of reaction is responsible for the cross-linking and stabilization of the collagen chains, which is effected by relatively small amounts of chromium.

A research grant from *Statens Tekniska Forskningsråd* is gratefully acknowledged. The author is also indebted to Miss Marianne Påhlman for her diligent assistance in the experimental work.

Garverinäringens forskningsinstitut, Stockholm.

REFERENCES

1. GUSTAVSON, K. H., Arkiv för Kemi, *11*, 185 (1957).
2. For a general outline, see GUSTAVSON, K. H., The Chemistry of Tanning Processes, pp. 11–58, Academic Press, New York, N.Y. (1956); further, J. Am. Leather Chem. Assoc. *48*, 559 (1953); Discussions Faraday Soc. No. 16, pp. 185–195 (1954).
3. GUSTAVSON, K. H., J. Colloid Sci. *1*, 397 (1946); Svensk Kem. Tidskr. *56*, 14 (1944); *58*, 2, 274 (1946); J. Soc. Leather Trades Chem. *30*, 264 (1946); *34*, 259 (1950); *35*, 160, 270 (1951); *36*, 182 (1952).
4. GUSTAVSON, K. H., J. Am. Leather Chem. Assoc. *47*, 151 (1952).
5. STIASNY, E., and GERGELY, E., Collegium, *1931*, 444; STIASNY, E., Gerbereichemie, p. 439. Th. Steinkopff, Dresden, 1931.
6. JANDER, G., and SCHEELE, W., Z. anorg. allg. Chem. *206*, 241 (1932); JANDER, G., and JAHR, K. F., Kolloid Beih. *41*, 1 (1934); *41*, 297 (1935); RIESS, C., and BARTH, K., Collegium, *1935*, 62.
7. GUSTAVSON, K. H., J. Soc. Leather Trades Chem, *35*, 270 (1951).
8. GUSTAVSON, H. K., Svensk Kem. Tidskr. *58*, 2 (1946).
9. GUSTAVSON, K. H., J. Colloid Sci. *1*, 397 (1946).
10. GUSTAVSON, K. H., J. Am. Leather Chem. Assoc. *21*, 559 (1926): see also STIASVNY, E., and BALÁNYI, D., Collegium, *1928*, 72; KÜNTZEL, A., *et al.*, ibid. *1934*, 261.
11. GUSTAVSON, K. H., J. Am. Leather Chem. Assoc. *22*, 60 (1927); *26*, 635 (1931); cf. MERRILL, H. B., NIEDERCORN, J. G., and QUARCK, R., ibid. *23*, 187 (1928).
12. BALÁNYI, D., Collegium, *1929*, 153: see also J. Am. Leather Chem. Assoc. *26*, 655 (1931).
13. BOWES, J. H., and KENTEN, R. H., Biochem. J. *43*, 358 (1948); BOWES, J. H., ELLIOTT, R. G., and Moss, J. A., ibid. *61*, 143 (1955). For a general account see GUSTAVSON, K. H., The Chemistry and Reactivity of Collagen, pp. 37–42, 102–113. Academic Press, New York, N.Y., 1956.
14. CASSEL, J. M., and MCKENNA, E., J. Am. Leather Chem. Assoc, *48*, 142 (1953); see also ref. (1).
15. WIEDERHORN, N. W., REARDON, G. W., and BROWNE, A. R., J. Am. Leather Chem. Assoc. *48*, 7 (1953); WRIGHT, B. A., and WIEDERHORN, N. W., J. Polymer Sci. *7*, 105 (1951); see also ref. (1).
16. NAIDUS, E. S., and BROWNE, A. R., J. Am. Leather Chem. Assoc. *50*, 546 (1955).
17. ZAHN, H., and WEGERLE, D., Das Leder *5*, 121 (1954).
18. WEIR, C. E., J. Am. Leather Chem. Assoc. *44*, 108 (1949); WEIR, C. E., and CARTER, J., J. Research Natl. Bur. Standards, *44*, 559 (1950).

Tryckt den 30 juli 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 46

Communicated 10 April 1957 by Arne Fredga

Optical resolution and configuration of α-phenylglutaric acid

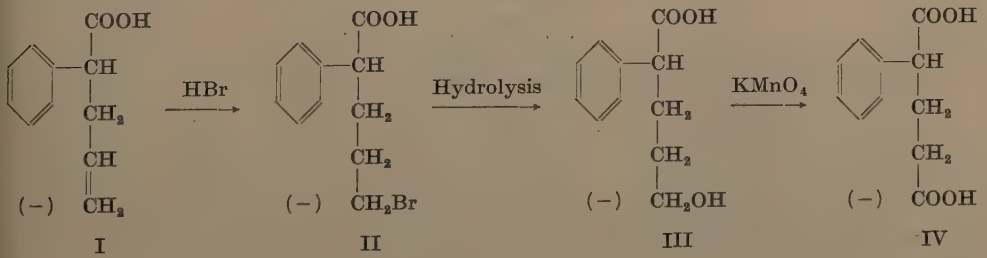
By Lars Westman

During current work on steric relationships it was desirable to obtain optically active α-phenylglutaric acid and determine its configuration. This acid is a valuable intermediate for the connection of the synthetic plant growth regulator 1,2,3,4-tetrahydro-1-naphtoic acid to the steric system of D-glyceraldehyde (1). For this reason the first named acid was resolved into its optical antipodes, as has been previously described in a short communication (2). A detailed account of the resolution is given in the present paper. In a previous paper Fredga and the present author have reported the connection of optically active allyl-phenylacetic acid to the glyceraldehyde system (3). Now this acid was used as the parent substance in the determination of the configuration of α-phenylglutaric acid by the chemical method.

Racemic α-phenylglutaric acid, prepared according to Horning and Finelli (4) and recrystallised first from formic acid and then from a mixture of ether and petroleum ether, melts at 100–102°. Fichter and Merckens (5) and Ansell and Hey (6) have reported a melting point of 82–84°. Their acids were recrystallised from benzene and benzene + light petroleum respectively. It seems that they had obtained an unstable modification. The resolution of the racemic acid could be performed through the secondary salts of quinine and brucine. Some other alkaloids also gave crystallised salts, but the alkaloids mentioned showed the best resolving power. Quinine in dilute ethanol gave the (+)-acid and brucine in methanol the (−)-acid in relatively good yields. On recrystallising the crude acids from formic acid both antipodes are obtained in a pure state. The melting point was 129–131° and the optical rotation found was $[\alpha]_D^{25} = +86.1$ and $[\alpha]_D^{25} = -85.5$ respectively (measured in abs. ethanol).

Configuration of α-phenylglutaric acid

In 1933 Linstead and Rydon (7) reported that allylacetic acid could be converted into δ-bromovaleric acid by addition of hydrogen bromide to the unsaturated acid dissolved in hexane.



I II III IV

Following the reaction scheme given above the present author transformed allyl-phenylacetic acid (I) to α-phenyl-δ-bromovaleric acid (II) and further to α-phenyl-glutaric acid (IV). This was performed both with racemic and (−)-acid (I). The (−)-α-phenylglutaric acid obtained showed an optical rotation in absolute ethanol of $[\alpha]_D^{25} = -82°4$, which indicates that almost maximal activity is maintained during the reactions. As (−)-allyl-phenylacetic acid gives (−)-α-phenylglutaric acid it is evident that allyl-phenylacetic acid and α-phenylglutaric acid with the same direction of rotation have the same configuration. The racemic intermediate (II) crystallised from formic acid in small, faintly yellow needles with melting point 84–85°. The (−)-acid (II) was too soluble in formic acid to be recrystallised from this solvent but from hexane + ether it was obtained in a state of purity. M.p. 52–54°. Optical rotation $[\alpha]_D^{25} = -54°4$ (in abs. ethanol). Racemic α-phenyl-δ-hydroxyvaleric acid (III) formed by hydrolysis of inactive (II) was not quite pure even after several recrystallisations from chloroform + petroleum ether. It was obtained as an amorphous powder, which melts at 79–81°. The equivalent weight found was about two per cent too high. The (−)-acid (III), not recrystallised, melts at 51–55° and has an optical rotation in ethanol solution of $[\alpha]_D^{25} = -44°7$.

Experimental

Racemic α-phenylglutaric acid

The anhydride was prepared according to Horning and Finelli (4). Hydrolysis of the anhydride with boiling water for 30 minutes followed by extraction of the cold solution with ether gave the crude crystallised acid, when the ether was evaporated. After recrystallisation from formic acid, it melted at 98–101°; further recrystallisation from ether + petroleum ether raised the m.p. to 100–102°.

0.1368 g of acid: 13.08 ml 0.1001 *N* NaOH.
Equiv. wt. calc. 104.1, found 104.5.

Preliminary experiments on resolution.—0.208 g (0.001 moles) of racemic acid and 0.002 moles of the bases were dissolved in ethanol or methanol. The solutions were allowed to stand and in the case of ethanol, water was added gradually until crystals were formed. The crystals were filtered off, the acid liberated and the optical activity measured in absolute ethanol. The results are given in Table 1.

Table 1.

Base	$[\alpha]_D^{25}$ of acid, measured in abs. ethanol	
	From ethanol + water	From methanol
Strychnine	− 3°	—
Cinchonine	+ 20°	+ 27°
Quinine	+ 53°	+ 35°
Brucine	− 5°	− 51°
Quinidine	± 0°	oil
Cinchonidine	+ 20°	− 21°

Optical resolution of α-phenylglutaric acid

33.5 g (0.161 moles) of racemic α-phenylglutaric acid and 104.0 g (0.322 moles) of quinine were dissolved in 800 ml of hot ethanol (96%). The solution was allowed to cool and crystallise for 24 hours in a refrigerator. The precipitate was filtered off, washed with a little cold ethanol, dried and weighed (76.0 g). From a sample (about 0.5 g) the acid was isolated and the optical activity measured in absolute ethanol. The remaining salt was recrystallised from dilute alcohol, and the process repeated until the optical activity of the acid remained constant. The course of the resolution is given in Table 2.

Table 2.

Cryst. No.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D^{25}$ of acid
	Ethanol	Water		
1	800	—	76.0	+52°
2	450	—	58.8	+72°5
3	450	10	51.5	+81°
4	250	15	46.3	+83°5
5	250	25	42.0	+84°
6	250	25	38.4	+86°
7	220	25	35.7	+86°
8	200	25	32.8	+86°

The mother liquor from the first crystallisation with quinine was evaporated to dryness at room temperature and the acid was liberated from the residue. This acid (13.8 g), which showed an optical activity of about −47° (in abs. ethanol), and 62.0 g of brucine were dissolved in 250 ml hot methanol and the solution was left in a refrigerator for 24 hours. The crystals formed were filtered off and washed with cold methanol. The progress of the resolution was followed as described above and the results are given in Table 3.

Table 3.

Cryst. No.	Methanol (ml)	Weight of salt (g)	$[\alpha]_D^{25}$ of acid
0	—	—	−47°
1	250	58.6	−72°
2	150	49.4	−76°
3	150	47.0	−77°
4	150	42.6	−82°
5	150	39.5	−82°5
6	150	36.7	−84°
7	150	32.4	−84°5
8	150	27.2	−84°5

(+)-α-*Phenylglutaric acid*

32.8 g of the quinine salt was decomposed with 2 *M* sulphuric acid and the organic acid thoroughly extracted with ether. The ether was removed at room temperature and the crude acid (7.4 g) was recrystallised once from pure formic acid. The acid forms colourless needles or rods with m.p. 129–131°.

0.0498 g of acid: 9.59 ml of 0.0496 *N* NaOH.
Equiv. wt. calc. 104.1, found 104.7.
0.1063 g of acid dissolved in abs. ethanol to 10.00 ml: $2\alpha_D^{25} = +1.83°$, $[\alpha]_D^{25} = +86°1$; $[M]_D^{25} = +179°0$.

Weighed amounts of acid were made up to 10.00 ml with different solvents and the optical activity measured. In the case of water the acid was neutralized with NaOH to pH 7. The results are given in Table 4.

Table 4.

Solvent	mg of acid	$2\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Acetone	80.5	+1°65	+102°5	+213°5
Ethyl acetate	81.5	+1°66	+101°8	+212°
Acetic acid	81.0	+1°51	+ 93°2	+194°
Chloroform	84.6	+1°47	+ 86°9	+181°
Ethanol	106.3	+1°83	+ 86°1	+179°
Water (ion)	81.6	+0°49	+ 30°0	+ 62°5

(−)-α-*Phenylglutaric acid*

27.2 g of the brucine salt was decomposed in the same way as described for the antipode. 4.5 g of the acid was obtained. It was recrystallised once from formic acid. M.p. 129–131°.

0.1014 g of acid: 9.74 ml of 0.1001 *N* NaOH.
Equiv. wt. calc. 104.1, found 104.0.
0.0889 g of acid dissolved in abs. ethanol to 10.00 ml: $2\alpha_D^{25} = -1°52$, $[\alpha]_D^{25} = -85°5$; $[M]_D^{25} = -178°0$.

Conversion of allyl-phenylacetic acids to α-phenylglutaric acids

Racemic α-phenyl-δ-bromovaleric acid

5.0 g of racemic allyl-phenylacetic acid [prepared from ethyl phenylcyanoacetate and allyl bromide according to Wideqvist (8) and Pickard and Yates (9)] was dissolved in 50 ml of hexane. The solution was kept in ice water and saturated with hydrogen bromide obtained from tetralin and bromine. In order to purify the gas before use it was washed with tetralin and passed over paraffin and calcium chloride. During the reaction the α-phenyl-δ-bromovaleric acid separated as a crystalline mass. The acid was filtered off, washed with cold hexane and dried. The yield of crude acid was

6.0 g (82%). It melted at 75–79°. After two recrystallisations from cyclohexane + hexane and one from formic acid the pure acid was obtained as small faintly yellow needles. M.p. 84–85°.

0.1764 g of acid: 6.84 ml 0.1001 *N* NaOH.
Equiv. wt. calc. 257.1, found 257.6.
Bromine: calc. 31.08 %, found 31.23 %.

(−)-α-Phenyl-δ-bromovaleric acid

3.5 g of (−)-allyl-phenylacetic acid (3) ($[\alpha]_D^{25} = -103°4$, in benzene) was dissolved in 35 ml of hexane. While cooling the solution in ice water hydrogen bromide was passed in and the (−)-α-phenyl-δ-bromovaleric acid formed was collected in the same way as described above. Yield 4.6 g. After three recrystallisations from hexane + ether the acid melted at 52–54°.

0.0638 g of acid: 5.04 ml of 0.0495 *N* NaOH.
Equiv. wt. calc. 257.1, found 255.7.
0.0983 g of acid dissolved in abs. ethanol to 10.00 ml: $2\,\alpha_D^{25} = -1°07$, $[\alpha]_D^{25} = -54°4$; $[M]_D^{25} = -140°0$.

Racemic α-phenyl-δ-hydroxyvaleric acid

2.8 g of racemic α-phenyl-δ-bromovaleric acid was dissolved in 70 ml of 0.2 *M* sodium carbonate solution. The solution was refluxed for 50 minutes. After cooling, the solution was acidified with dilute sulphuric acid and extracted three times with ether. The ether layers were combined and the ether removed first at room temperature, and finally in vacuo. The colourless residue soon solidified, yielding 2.0 g of crude acid. After recrystallisation twice from chloroform and twice from chloroform + petroleum ether a colourless powder remained. M.p. 79–81°.

0.0732 g of acid: 7.84 ml 0.0495 *N* NaOH.
Equiv. wt. calc. 194.2, found 197.7.

The equivalent weight indicates that the acid is not quite pure.

(−)-α-Phenyl-δ-hydroxyvaleric acid

3.0 g of (−)-α-phenyl-δ-bromovaleric acid ($[\alpha]_D^{25} = 54°4$, in abs. ethanol) was dissolved in 70 ml of 0.2 *M* sodium carbonate solution. The solution was refluxed for 40 minutes (oil bath, 120°). After cooling, the acid was liberated as has been described for the racemic hydroxy acid. It remained as a viscous mass but after standing overnight in a refrigerator (−13°) it crystallised, yielding 2.2 g of crude acid, which melted at 51–55°. The acid was not further purified. $[\alpha]_D^{25} = -44°7$, measured in abs. ethanol.

Racemic α-phenylglutaric acid

2.0 g of racemic α-phenyl-δ-hydroxyvaleric acid was dissolved in 20 ml of 2.5 *M* sodium hydroxide solution. While stirring the solution a saturated potassium permanganate solution was added. The oxidation mixture, which was kept at room temperature, at first had a green colour indicating the formation of manganate. After adding permanganate until the mixture showed a lasting violet colour the

manganate and the excess of permanganate were reduced to manganese dioxide with sodium sulphite. The manganese dioxide was filtered off, the filtrate acidified with dilute sulphuric acid and carefully extracted with ether. On removing the ether from the combined extracts first at room temperature, and finally in vacuo, a viscous faintly brownish mass remained. The viscous mass solidified when seeded with α-phenylglutaric acid, yielding 1.4 g of crude acid. It was recrystallised from pure formic acid. Yield 0.45 g. M.p. 100–102°.

0.1537 g of acid: 14.60 ml 0.1001 *N* NaOH.
Equiv. wt. calc. 104.1, found 104.7.

An authentic specimen of α-phenylglutaric acid melts at 100–102°; no depression was found with a mixed sample.

(−)-α-Phenylglutaric acid

2.2 g of (−)-α-phenyl-δ-hydroxyvaleric acid ($[\alpha]_D^{25} = -44°7$, in abs. ethanol) was dissolved in a solution of 2.0 g sodium hydroxide in 20 ml of water. The solution was kept at room temperature and the oxidation carried out as described above. When the oxidation was finished and the mixture worked up as before a crystalline mass of crude optically active α-phenylglutaric acid was obtained. Yield 1.8 g. The acid was recrystallised twice from formic acid. Yield 0.9 g. M.p. 128–130°.

0.0507 g of acid: 9.82 ml 0.0495 *N* NaOH.
Equiv. wt. calc. 104.1, found 104.3.
0.1116 g of acid dissolved in abs. ethanol to 10.00 ml: $2\,\alpha_D^{25} = 1°84$, $[\alpha]_D^{25} = -82°4$.

The rotatory power of the pure (−)-α-phenylglutaric acid in abs. ethanol is $[\alpha]_D^{25} = -85°5$. The value obtained indicates a slight racemisation during the reactions.

SUMMARY

α-Phenylglutaric acid (IV) has been resolved into optical antipodes. Optically active α-phenylglutaric acid is also obtained from allyl-phenylacetic acid (I) with the same direction of rotation. Since the asymmetric carbon atom is not involved in the reactions and the configuration of the latter acid is known, the α-phenylglutaric acid has been related to the glyceraldehyde system. During the investigation racemic and (−)-α-phenyl-δ-bromovaleric acid (II) together with racemic and (−)-α-phenyl-δ-hydroxyvaleric acid (III) have been prepared as intermediates. Thus the configurations of these acids have also been determined.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professor Arne Fredga for suggesting the present work and for valuable discussions. A grant from the *Swedish Natural Science Researh Council* is gratefully acknowledged.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, April 1957.

REFERENCES

1. WESTMAN, L., to be published.
2. FREDGA, A., and WESTMAN, L., Acta Chem. Scand. *10*, 477 (1956).
3. FREDGA, A., and WESTMAN, L., Arkiv Kemi *7*, 193 (1954).
4. HORNING, E. C., and FINELLI, A. F., J. Amer, Chem. Soc. *71*, 3204 (1949).
5. FICHTER, F., and MERCKENS, O., Ber. *34*, 4147 (1902).
6. ANSELL, M. F., and HEY, D. H., J. Chem. Soc. *1950*, 1683.
7. LINSTEAD, R. P., and RYDON, H. N., Nature *132*, 643 (1933).
8. WIDEQVIST, S., Arkiv Kemi Mineral. Geol. *24 B*, nr. 14 (1947).
9. PICKARD, R. H., and YATES, J., J. Chem. Soc. *95*, 1011 (1909).

Tryckt den 30 juli 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 47

Communicated 10 April 1957 by Arne Fredga and Karl Myrbäck

Stereochemical studies. II

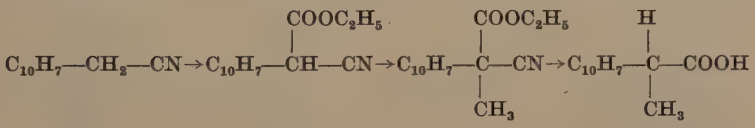
The enantiomers of α-(3-thianaphthenyl)-propionic acid

By Berndt Sjöberg

With 10 figures in the text

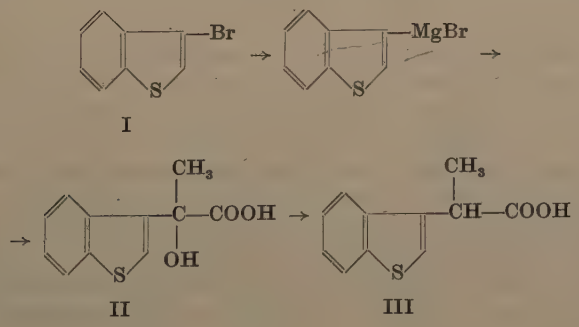
As a part of the stereochemical studies of aralkyl carboxylic acids outlined earlier (1), the author has prepared, resolved and established the configuration of the enantiomers of α-(3-thianaphthenyl)-propionic acid (III). In addition to the interest this acid has for rotatory dispersion studies, the investigation of the growth regulating properties of the enantiomers may give further information about the connection between the structure and auxin activity of similar compounds. Work in this field has recently been reviewed by Fredga (2).

For the preparation of racemic α-(3-thianaphthenyl)-propionic acid (III) two different methods were tried. The corresponding naphthyl-propionic acids (1, 3) were prepared according to the following general procedure described by Wideqvist (4):



However, in going from thianaphthene to α-(3-thianaphthenyl)-propionic acid by a similar scheme the yield was low (10%), and so an alternative way was chosen.

Selective addition of a Grignard reagent to the keto group of a keto acid or keto ester is possible. The hydroxy acid formed can be reduced, for instance, with stannous chloride–hydrochloric acid. The desired α-(3-thianaphthenyl)-propionic acid (III) was prepared according to a procedure indicated by the following scheme:



Lapkin and Golovkova reacted the Gringard reagent of 1-bromonaphthalene with ethyl pyruvate in refluxing ether, and after saponification they obtained α-hydroxy-α-(1-naphthyl)-propionic acid in 35% yield (5). The corresponding reaction sequence with 3-bromo-thianaphthene gave the best yield (50%) when the temperature was maintained below 0° during the addition of the Grignard reagent to the ethyl pyruvate. The ethyl pyruvate was prepared by a method similar to that described in Organic Syntheses for the methyl ester (6). The α-hydroxy-α-(3-thianaphthenyl)-propionic acid (II) was reduced with stannous chloride–hydrochloric acid with acetic acid as solvent.

The resolution of the racemic acid III could be performed with cinchonine and cinchonidine. The dextrorotatory acid was obtained by recrystallization of the chinchonine salt from dilute ethanol. The cinchonidine salt gave the (−)-form from the same solvent. The melting point of the active acids was 64–66°.

The rotatory power was determined in different solvents. The activity data of α-(3-thianaphthenyl)-propionic acid and α-(1-naphthyl)-propionic acid are given in Table 1. The two acids show a similar difference in molecular rotation from one solvent to another, indicating that the acids with the same direction of rotation also have the same configuration (7).

Table 1. $[M]_D^{25}$ for (−)-α-(3-thianaphthenyl)-propionic acid (TP) and (+)-α-(1-naphthyl)-propionic acid (NP).

Solvent	$[M]_D^{25}$ TP	NP
Benzene	−226.1°	361.5°
2.2.4-Trimethylpentane	−215°	342.4°
Acetic acid	−206.5°	304.8°
Acetone	−190.2°	292.1°
Chloroform	−171.2°	268.2°
Ethanol	−165.5°	240.9°
Water (Na-salt)	− 2.3°	69.9°

A definite proof for the sterical relations was obtained from the melting point diagrams. For the task of relating the antipodes of III to a substance of known configuration, comparison of one of these compounds with the antipodes of α-(1-naphthyl)-propionic acid by the Fredga method (8) seemed to be possible. The (+)-form of α-(1-naphthyl)-propionic acid has been related to the D-glyceraldehyde system (3). α-(Naphthyl)-propionic acid has a remarkably stable racemate (Fig. 7), whose melting point is 80° higher than that of the antipodes. The maximum completely dominates the diagram and no eutectic point could be established with certainty from the melting points. However, that the maximum was a true racemic compound could be shown from the X-ray powder photograms and from infrared absorption spectra. The infrared spectra are reproduced in Figs. 1 and 2.

Previous work on the infrared absorption of stereoisomeric compounds, among others by Eliel, Kofron (9) and Rosenberg, Schotte (10, 11) has shown that significant differences can be observed between the racemic form and the antipodes. In Figs. 1 and 2 the absorption curves of the racemic α-(1-naphthyl)-propionic acid

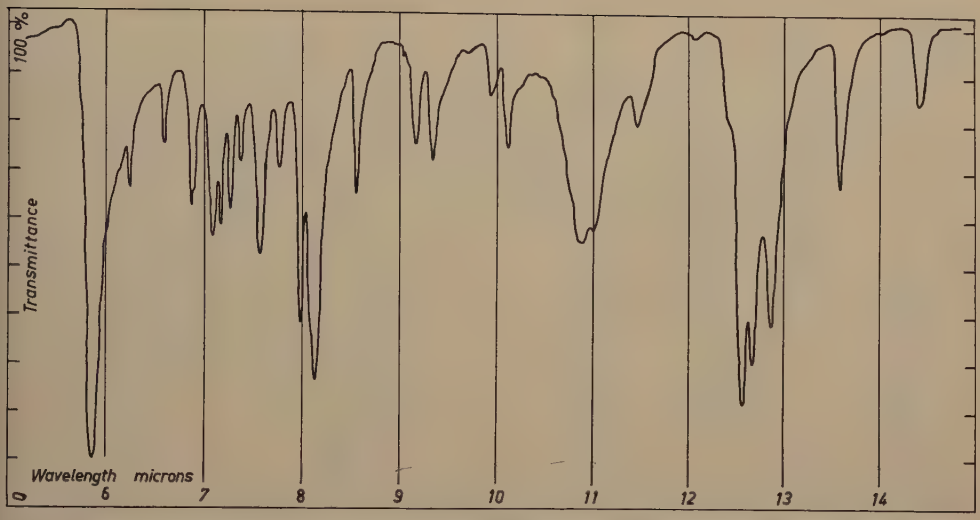


Fig. 1. IR absorption spectrum of racemic α-(1-naphthyl)-propionic acid. NaCl prism, KBr phase.

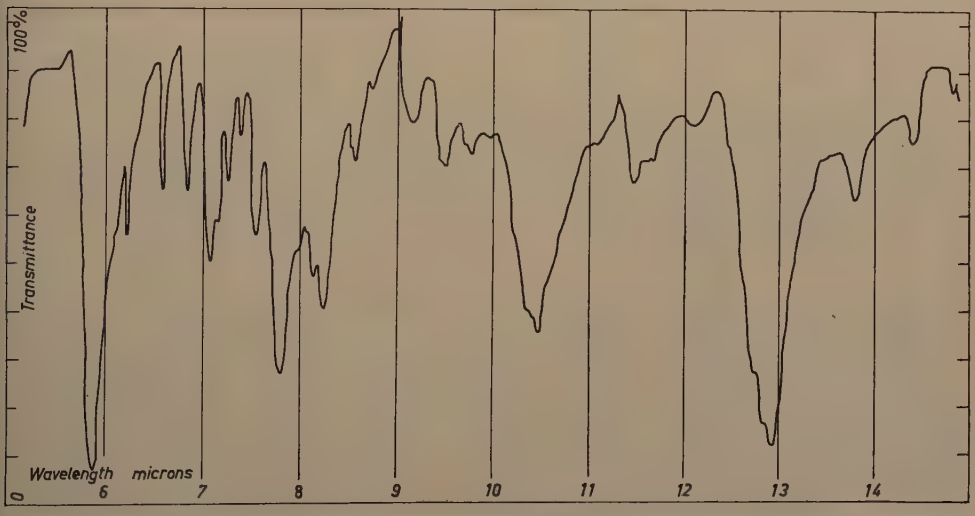


Fig. 2. IR absorption spectrum of (−)-α-(1-naphthyl)-propionic acid. NaCl prism, KBr phase.

and its antipode are reproduced. It is seen that the carbonyl stretching peak for the racemic compound occurs at 5.86 μ and the corresponding peak for the antipode at 5.89 μ. The band ascribed to the bendings of OH is for the racemic compound situated at 10.9–11.0 μ and for the antipode at 10.4–10.5 μ. These shifts of the absorption frequencies indicate weaker intermolecular hydrogen bonds in the racemate than in the antipode. However, the melting point of the racemate is 80° higher than that of the antipode. This fact ought therefore to be ascribed to different packing of the molecules of the racemate and the antipodes.

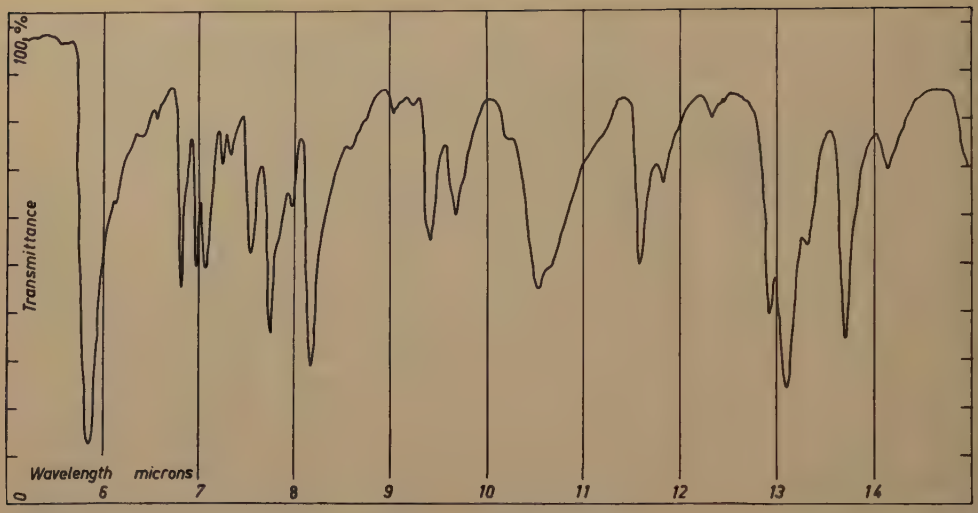


Fig. 3. IR absorption spectrum of racemic α-(3-thianaphthenyl)-propionic acid. NaCl prism, KBr phase.

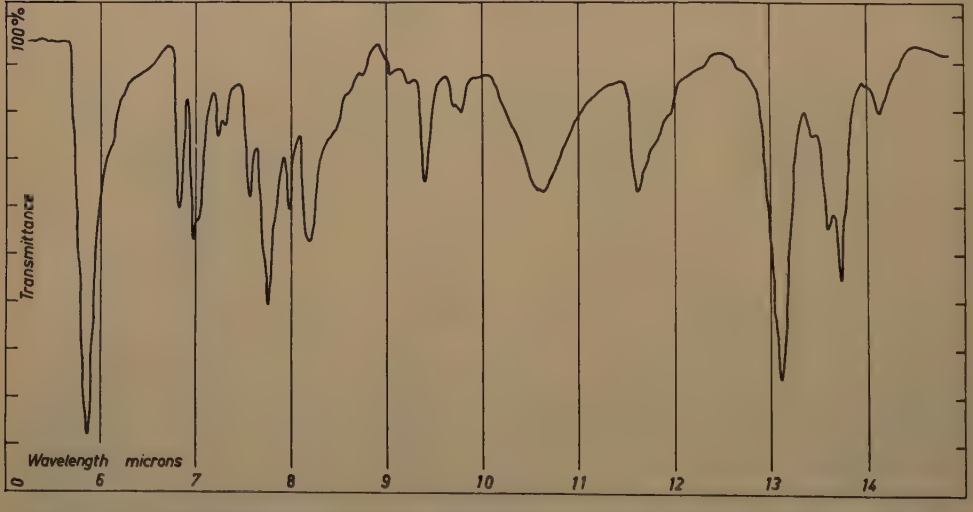


Fig. 4. IR absorption spectrum of (−)-α-(3-thianaphthenyl)-propionic acid. NaCl prism, KBr phase.

Further interesting differences between the absorption curve of the racemic α-(1-naphthyl)-propionic acid and that of its antipode can be observed. In the case of the racemic compound there are two sharp peaks, a small one at 7.77 μ and a bigger one at 7.98 μ. In the active form these are transformed into a single large peak at 7.81 μ and a shoulder at 7.89 μ. In the same way three peaks are observed for the racemic compound at 12.56 μ, 12.67 μ and 12.87 μ. For the antipode there is only one peak in the same region at 12.93 μ with two shoulders at 12.74 μ and 12. 85 μ. Differences of

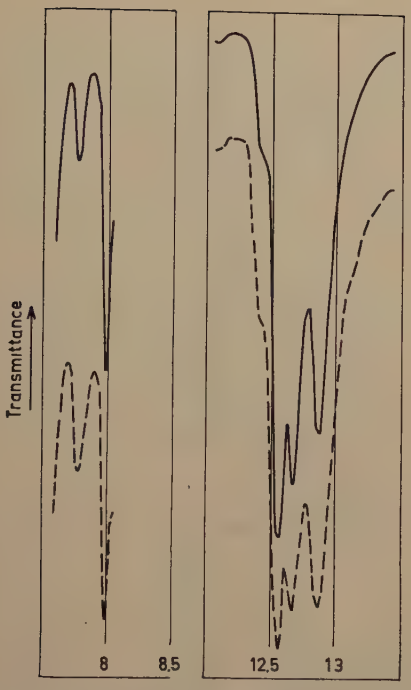


Fig. 5. IR absorption spectra between 7.5–8.5 μ and 12.0–13.5 μ for racemic α-(1-naphthyl)-propionic acid (solid curve); for a preparation containing 80 % of the racemate and 20 % of the antipode (dashed curve). NaCl prism, KBr phase.

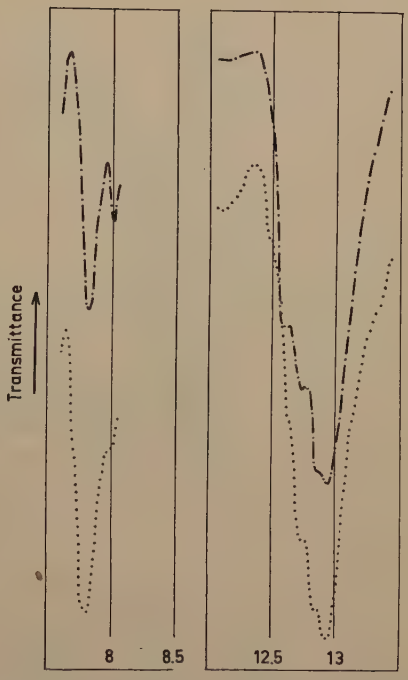


Fig. 6. IR absorption spectra between 7.5–8.5 μ and 12.0–13.5 μ for a preparation containing 80 % of (−)-α-(1-naphthyl)-propionic acid and 20 % racemic acid (dash-dotted curve); for (−)-α-(1-naphthyl)-propionic acid (dotted curve). NaCl prism, KBr phase.

this kind might perhaps in special cases be useful for determining the percentage of racemic and active form in a mixture. Measurements of the rotatory power or of the melting points are usually the simplest methods for this determination. However, if the rotatory power is low or if the melting points are unreliable (e.g. if the difference in melting points is too small) then the infrared absorption curves might be useful. As is seen in Fig. 5 there is a marked difference in the infrared absorption at these wavelengths between the racemic compound (m.p. 149.5°) and a preparation containing 80% of the racemate and 20% of the antipode (m.p. 148°).

The α-(3-thianaphthenyl)-propionic acid has a smaller tendency for racemate formation (Figs. 7 and 8) than the corresponding 1-naphthyl compound. Furthermore, the differences between the infrared absorption spectra of the racemate and the antipode of α-(3-thianaphthenyl)-propionic acid are considerably less than those encountered in the case of α-(1-naphthyl)-propionic acid (Figs. 3 and 4).

It has been shown that the possibility for two compounds to form a quasi-racemate is dependent on the racemate-formation tendency of the two components and on their structural similarities (8, 12, 13). Polar effects might also have a great influence on quasi-racemate formation. Now α-(1-naphthyl)-propionic acid has an extremely great racemate-formation tendency. This tendency is less for α-(3-thianaphthenyl)-

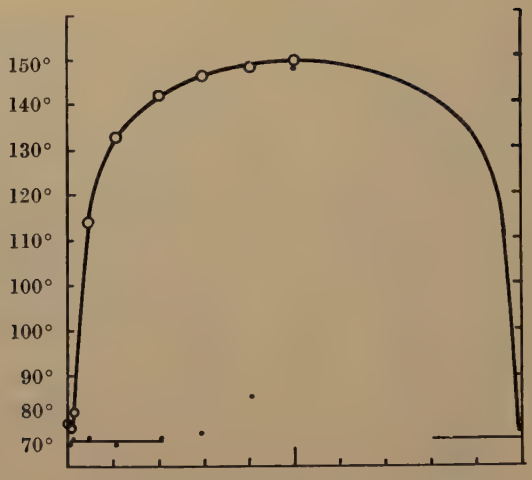


Fig. 7. Mole- % (+)-acid.
(+)- and (−)-α-(1-naphthyl)-propionic acid.

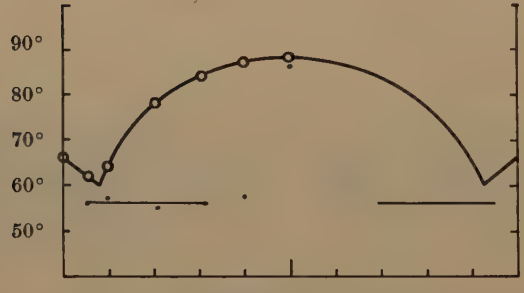


Fig. 8. Mole- % (+)-acid.
(+)- and (−)-α-(3-thianaphthenyl)-propionic acid.

propionic acid, but it is great compared to that of carboxylic acids in general. As the structural differences between these two acids are probably not too great (15), it seemed justified to compare them by the Fredga method.

By thermal analysis according to Kofler (14) it was shown with the aid of the contact method that a molecular compound was formed between (−)-α-(3-thianaphthenyl)-propionic acid and (+)-α-(1-naphthyl)-propionic acid. After the preliminary experiments the whole curve was determined for this two-component system. As is seen in Fig. 9, a very pronounced quasi-racemic compound is formed from the components in the ratio 1:1. The melting point curve for the system containing acids with the same direction of rotation (Fig. 10) shows mutual solubility in the solid state which is evidently due to structural similarity.

From the results obtained the conclusion can be drawn that α-(1-naphthyl)-propionic acid and α-(3-thianaphthenyl)-propionic acid with the same direction of rotation (measured in ethanol at the wave-length of the D-line) also have the same configuration. The dextrorotatory α-(1-naphthyl)-propionic acid has the configuration

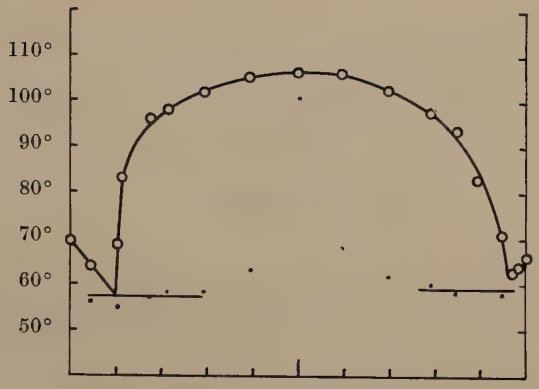


Fig. 9. Mole- % (−)-thianaphthenyl-acid.
(−)-α-(3-thianaphthenyl)-propionic acid and (+)-α-(1-naphthyl)-propionic acid.

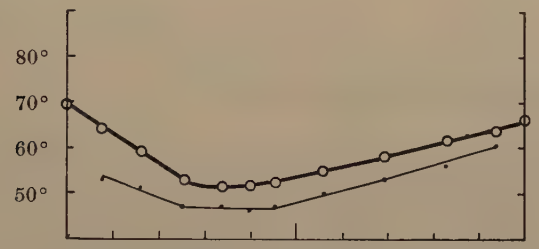
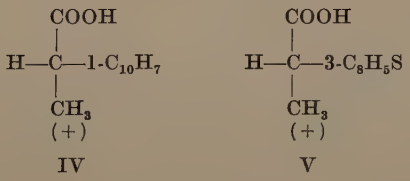


Fig. 10. Mole- % (−)-thianaphthenyl-acid.
(−)-α-(3-thianaphthenyl)-propionic acid and (−)-α-(1-naphthyl)-propionic acid.

Consequently (+)-α-(3-thianaphthenyl)-propionic acid has a stereoformula corresponding to V.



IV (3). If the thianaphthenyl group is considered as a substituent on the aliphatic carbon chain, this acid must be assigned to the D-series (15).

The plant growth regulating properties of α-(3-thianaphthenyl)-propionic acid have been investigated by Professor B. Åberg. The activity of the (+)-acid is rather strong and comparable with the activity of 3-indolyl-acetic acid (heteroauxin). In the *Avena straight growth test*, the (+)-form is about 100 times as active as the (−)-form. The (+)-antipode is about 30 times more active than the (−)-acid in *inhibiting the root growth of wheat* while the difference in the *flax root test* is about 3 times (16). A great number of optically active growth regulators of the general structure Ar–X–CH(R)–COOH have been investigated by Matell (17) and the D-form has been shown to

have the greater auxin activity. For acids of the type Ar–CH(R)–COOH the D-form also seems to have the greater activity (2). Of the acids investigated by Matell only the D-form had positive auxin activity but both antipodes of α-(3-thianaphthenyl)-propionic acid are active though with a marked difference between the D- and the L-form. This is also the case of α-(1-naphthyl)-propionic acid (3) and α-(3-indolyl)-propionic acid (16).

Experimental

Ethyl pyruvate

A solution of 88 g (1 mole) of freshly distilled pyruvic acid, 140 g (3 moles) of absolute ethanol, 350 ml of benzene and 0.2 g of *p*-toleuenesulfonic acid was placed in a flask supplied with water-separator. The solution was refluxed for 12 hours and then fractionated in a column. The yield of ester boiling at 44–46/15 mm was 82 g (71%).

3-Bromothianaphthene (I)

This substance was prepared according to Komppa (18) in a yield of 85%.

α-Hydroxy-α-(3-thianaphthenyl)-propionic acid (II)

In a three-necked flask equipped with a reflux condenser, a mechanical stirrer and a separatory funnel were placed 3.4 g (0.14 mole) of dry magnesium turnings and 50 ml of sodium-dried ether. A solution of 21.3 g (0.1 mole) of 3-bromothianaphthene in 60 ml ether was added during a period of 1 hour. The mixture was then refluxed for 3 hours. The Grignard reagent collected as a heavy oil in the bottom of the flask, but was dissolved when 50 ml dry benzene was added. The Grignard reagent was cooled and then added dropwise to a solution of 14 g (0.12 mole) ethyl pyruvate in 50 ml dry ether. The reaction vessel was cooled in a freezing mixture. Each drop of the Grignard reagent reacted vigorously with the ester causing separation of a white precipitate. The mixture was kept cold for 3 hours after the addition and then stirred for a further 3 hours at room temperature. The mixture was then hydrolysed with ice and dilute acetic acid. The organic layer was separated and the water extracted with ether. The combined ether extracts were transferred to a flask, the ether evaporated and the residue saponified with 10% potassium hydroxide. The alkaline water solution was extracted with ether. On acidifying the waterlayer the acid separated as an oil which soon solidified. The product was recrystallized from carbon tetrachloride forming long needles containing half a mole of solvent per mole of acid; yield 15 g (50%). The solvent-containing acid melted at 85–90°, crystallised again at 95–100° and had a definite melting point of 112–114°. Even when kept in vacuum at 50° for 24 hours the acid was not quite free from solvent. For analysis, a sample was recrystallized from water and dried over phosphorous pentoxide; m.p. 112–114°.

Anal. $C_{11}H_{10}O_3S$, $\frac{1}{2}$ CCl_4 (299.17):
Calc. equiv. wt. 299.2; C 46.16; H 3.37; S 10.72; Cl 23.7.
Found equiv. wt. 292.0; C 47.65; H 3.46; S 10.82; Cl 18.6.
$C_{11}H_{10}O_3S$ (222.27):
Calc. equiv. wt. 222.3; C 59.38; H 4.53; S 14.43.
Found equiv. wt. 223.5; C 58.65; H 4.53; S 13.97.

α-(3-Thianaphthenyl)-propionic acid (III)

A mixture of 22.2 g (0.10 mole) of II, 50 g (0.22 mole) of stannous chloride dihydrate and 200 ml of acetic acid was put in a 3-necked flask fitted with stirrer and an inlet-tube for hydrogen chloride. A stream of hydrogen chloride was passed into the mixture which was stirred continuously and maintained at 20° by cooling. The stream of hydrogen chloride was continued until a drop of the mixture no longer became red when treated with concentrated sulphuric acid (5 hours). The acetic acid was removed *in vacuo*, the residue dissolved in dilute sodium hydroxide and the alkaline solution extracted with ether. When the water solution was acidified with hydrochloric acid, the organic acid precipitated as an amorphous powder. The acid was recrystallized from formic acid and from ligroin (b.p. 85–110°).

Yield 10.5 g (51%), m.p. 86.5–88°.

Anal. $C_{11}H_{10}O_2S$ (206.27):
Calc. equiv. wt. 206.3; C 64.04; H 4.88; S 15.54.
Found equiv. wt. 206.1; C 64.16; H 4.84; S 15.46.

Resolution of the racemic acid

Preliminary experiments were carried out with 0.001 mole of the racemic acid and 0.001 mole of the alkaloids in different solvents. From the crystals formed the acid was liberated and the optical activity measured in absolute ethanol. The results are given in Table 2.

In a hot solution of 160 ml of ethanol and 80 ml of water 8.25 g (0.04 mole) of racemic acid and 11.78 g (0.04 mole) of cinchonidine were dissolved and left to crystallize at room temperature and then in a refrigerator. The salt was filtered off after 24 hours. The acid was isolated from a sample and the optical activity measured in absolute ethanol. The remaining salt was recrystallized from dilute alcohol (Table 3).

The mother liquors from the two first crystallizations were evaporated to dryness at room temperature and 4.55 g acid isolated with $[\alpha]_D^{25} = +52°$ (absolute ethanol). This acid was dissolved with 6.50 cinchonine in 20 ml of boiling ethanol and 5 ml of hot water was added. The salt obtained was recrystallized in the same way as the cinchonide salt (Table 4).

(−)-α-(3-Thianaphthenyl)-propionic acid

The acid was liberated from 6.3 g of the cinchonidine salt with dilute sulphuric acid and extracted with ether. It was recrystallized from ligroin (85–110°). Yield 2.1 g of acid forming small prisms with m.p. 64–66°.

Anal. $C_{11}H_{10}O_2S$: Equiv. wt. calc. 206.3; found 206.8.

Optical activity in different solvents

Weighed amounts of the acid were dissolved in the solvents, made up to a volume of 10.00 ml and the rotatory power determined. The results are given in Table 5.

(+)-α-(3-Thianaphthenyl)-propionic acid

The cinchonine salt was decomposed with dilute sulphuric acid and the organic acid extracted with ether. The yield of the crude acid was 2.0 g and after three recrystallizations from ligroin there remained 1.5 g with m.p. 64–66°.

Table 2. Preliminary tests on the resolution of α-(3-thianaphthenyl)-propionic acid.

Base	$[\alpha]_D^{25}$ cf acid in abs. ethanol			
	From methanol	From ethanol	From acetone	From ethyl acetate
Cinchonine	+ 3°	+25°	−14°	—
Cinchonidine	−43°	−44°	−43°	—
Quinine	± 0	−20°	—	—
Quinidine	± 0	± 0	—	—
Brucine	oil	oil	oil	oil
Strychnine	oil	oil	oil	oil
Morphine	oil	oil	oil	oil
(+)-α-Phenylethylamin	oil	oil	oil	oil
(+)-α-(2-Naphthyl)-ethylamin	+10°	+11°	—	—

Table 3. Recrystallization of the cinchonidine salt of α-(3-thianaphthenyl)-propionic acid.

Cryst. no.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D^{25}$ of acid
	Ethanol	Water		
1	160	80	10.9	−54°
2	120	60	8.5	−77°
3	80	40	7.3	−80°
4	60	30	6.5	−80°

Table 4. Recrystallization of the cinchonine salt of α-(3-thianaphthenyl)-propionic acid.

Cryst. no.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D^{25}$ of acid
	Ethanol	Water		
1	20	5	7.3	+77°
2	10	3	6.2	+78.5°
3	10	3	5.0	+78.5°

Table 5. $[\alpha]_D^{25}$ and $[M]_D^{25}$ of (−)-α-(3-thianaphthenyl)-propionic acid.

Solvent	mg of acid	$2\,\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Benzene	72.1	−1.58°	−109.6°	−226.1°
2.2.4-Trimethylpentane	17.8	−0.37°	−104°	−215°
Acetic acid	69.4	−1.39°	−100.1°	−206.5°
Acetone	73.2	−1.35°	− 92.2°	−190.2°
Chloroform	83.1	−1.38°	− 83.0°	−171.2°
Ethanol	75.4	−1.21°	− 80.2°	−165.5°
Water (neutr.)	94.1	−0.02°	− 1°	− 2°

Anal. $C_{11}H_{10}O_2S$: Equiv. wt. calc. 206.3; found 207.1.
0.0756 g acid dissolved in absolute ethanol to 10.00 ml: $2\alpha_D^{25} = +1.21°$, $[\alpha]_D^{25} = +80.0°$, $[M]_D^{25} = +164.8°$.

Infrared absorption measurements

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prism was used for the infrared absorption measurements. The samples were examined as solid pressed potassium bromide plates which were so prepared as to have comparable concentration.

Melting point diagrams

Weighed quantities of the components were dissolved in a few drops of pure acetone. The solvent was evaporated and the residue carefully powdered. All melting points were determined with the aid of a hot stage microscope according to Kofler (14). All preparations were stable for three subsequent meltings.

SUMMARY

α-Hydroxy-α-(3-thianaphthenyl)-propionic acid has been prepared and reduced to α-(3-thianaphthenyl)-propionic acid. The latter acid has been resolved into the optically active forms using cinchonidine and cinchonine. The rotatory power found was $[\alpha]_D^{25} = +80.2°$ and $[\alpha]_D^{25} = -80.0°$ respectively (in absolute ethanol). The optical activity has been determined in different solvents and compared with the rotatory power of α-(1-naphthyl)-propionic acid in the same solvents.

The two-component systems of (−)-α-(3-thianaphthenyl)-propionic acid and the antipodes of α-(1-naphthyl)-propionic acid have been investigated. The acids with opposite direction of rotation give a quasi-racemic compound, indicating that they also have opposite configuration.

The infrared spectra have been investigated.

The possibility of using differences in the spectra to determine the extent of racemization has been discussed.

Preliminary experiments on the auxin effect of the enantiomers of α-(3-thianaphthenyl)-propionic acid are reported.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Professor Arne Fredga for his kind interest in this work and for samples of the antipodes of α-(1-naphthyl)-propionic acid. Thanks are also due to Professor Börje Åberg, Royal Agricultural College, Uppsala, for kindly reporting the preliminary results on the growth regulating activity.

I am also indebted to Dr. Sixten Abrahamsson for the X-ray investigations, to Dr. Andreas Rosenberg for valuable discussions, and to Mr. Stig Bergwall for experimental help with the infrared work.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, April 1957.

REFERENCES

1. SJÖBERG, B., Arkiv Kemi *9*, 295 (1956).
2. FREDGA, A., Festschrift Arthur Stoll, Basel 1957, p. 795.
3. FREDGA, A., Arkiv Kemi *8*, 463 (1955).
4. WIDEQVIST, S., Arkiv Kemi, Mineral. Geol. *24 B*, N:r 14 (1947).
5. LAPKIN, I. I., and GOLOVKOVA, A. I., J. Gen. Chem. (U.S.S.R.) *18*, 485 (1948); Chem. Abstr. *42*, 7273 (1948).
6. Organic Syntheses, Coll. Vol. III, 610 (1955).
7. FREDGA, A., Studien über Selen-di-karbonsäuren und Diselen-di-karbonsäuren, Diss. Uppsala 1935, p. 184. (Also in Uppsala Universitets Årsskrift 1935:5, p. 184.)
8. FREDGA, A., "The Svedberg 1884 30/8 1944", Uppsala 1944, p. 261.
9. ELIEL, E. L., and KOFRON, J. T., J. Am. Chem. Soc. *75*, 4585 (1953).
10. SCHOTTE, L., and ROSENBERG, A., Arkiv Kemi *8*, 551 (1955).
11. SCHOTTE, L., Arkiv Kemi *9*, 397 (1956).
12. MATELL, M., Arkiv Kemi *5*, 17 (1952).
13. PETTERSSON, K., Arkiv Kemi *10*, 308 (1956).
14. KOFLER, L., and KOFLER, A., Mikro-Methoden, Innsbruck 1948.
15. SJÖBERG, B., Acta Chem. Scand. *10*, 1192 (1956).
16. ÅBERG, B., private communication.
17. MATELL, M., Stereochemical Studies on Plant Growth Substances, Diss. Uppsala 1953, p. 30.
18. KOMPPA, G., J. prakt. Chem. *122*, 319 (1929).

Tryckt den 12 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 48

Communicated 8 May 1957 by Sven Hörstadius and Per Eric Lindahl

Turnover rate of phosphate in mitochondrial thiamine diphosphate

By Karl-Heinz Kiessling

With 2 figures in the text

The phosphate turnover in thiamine phosphates has been studied in a mitochondrial system (1). The intramitochondrial thiamine diphosphate[1] was shown to incorporate radioactive phosphate more rapidly when a substrate like pyruvate was metabolized than without added substrate. Synthetic thiamine phosphates added to the mitochondria did not take part in the phosphate turnover to any appreciable extent.

In the present note the incorporation rate of radioactive phosphate in intramitochondrial TDP has been studied and compared with that in ATP–ADP.

Experimental

Mitochondria from guinea-pig liver were prepared mainly according to Schneider and Hogeboom (2), and suspended in 1.5 ml 0.5 *M* phosphate buffer pH 7.4 and 0.25 *M* sucrose to a final volume of 40 ml. Into a Vernbach flask were introduced 20 mg ADP, 1.0 ml 0.1 % cytochrome C, 0.3 mg cozymase, 0.3 ml 0.3 *M* α-ketoglutarate, 1.5 ml 0.3 *M* pyruvate, 1.0 ml 0.1 *M* $MgCl_2$, 300 mg glucose, radioactive inorganic phosphate, and hexokinase in excess. The flask was cooled to 0°C and mitochondria were added, the final volume being 51 ml. Two millilitres from the Vernbach flask were transferred to a Warburg vessel with 0.3 ml 2 *M* KOH in the center well and air in the gas space. A similar incubation was carried out in another flask, with 0.25 *M* sucrose in place of the same volume of α-ketoglutarate and pyruvate. Samples (15 ml) were taken after 0, 7, and 20 minutes, the proteins precipitated with ice-cold TCA (final concentration 9%), and after ten minutes at 0°C pH was adjusted to 3. One millilitre from each sample was taken for isolation of ATP–ADP, and to the remainder 3 mg synthetic TDP was added in order to facilitate the isolation of the intramitochondrial TDP. The combined TDP (synthetic + intramitochondrial) was adsorbed on 3 g of Fuller's earth, eluated with 15 ml pyridine–water (1:2), and the pyridine removed with ether. After the addition of four volumes of ethanol and one ml of 1 *M* $Ba(Ac)_2$, the samples were kept at 0°C for two hours, and the precipitate centrifuged down. The precipitate was then dissolved in 2 ml 0.1 *M* HCl, insoluble rests discarded, and Ba^{++} removed with H_2SO_4. Then pH was adjusted to 3, and the volume made up

[1] *Abbreviations:* TDP, thiamine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; P_i, inorganic phosphate; TCA, trichlorcacetic acid.

to 5 ml with H_2O. From this solution 2 ml were spread as a line on a Whatman paper 1, and TDP isolated according to Kiessling (3). The TDP obtained in this way was purified a second time with one of the chromatography methods of Siliprandi and Siliprandi (4). Controls gave evidence that no inorganic phosphate contaminated the TDP. After 10 minutes hydrolysis in 1 *M* HCl the specific radioactivity was determined. The method of Berenblum and Chain (5) was used for the determination of phosphorus, and the radioactivity was measured in an "M 6 liquid counter tube" from 20th Century Electronics Ltd. The radioactivity figures refer to counts per 5 minutes. The TDP was supplied by Roche Products Ltd., and ADP in the form of the sodium salt had been purchased from Sigma Chemical Company.

The yeast hexokinase was prepared at the Wenner Gren Institute in Stockholm according to Berger *et al.* (6), except for the cytolysis in toluene, which was performed as described by Bailey and Webb (7). The purification was followed up to step five.

ATP–ADP was precipitated with Lohmann's reagent according to Carter (8), and inorganic phosphate removed by a paper chromatographic solvent described by Siliprandi and Siliprandi (4). In this solvent inorganic phosphate moves easily, but ATP–ADP hardly at all.

Results and discussion

In a previous paper (1) intramitochondrial TDP was found to incorporate radioactive phosphate. After 20 minutes incubation with pyruvate as a substrate the radioactivity of the hydrolyzable phosphate was $\frac{1}{2}$–$\frac{2}{3}$ of that of ATP–ADP.

In the present experiments the incorporation rate of phosphate in TDP has been compared with that in ADP–ATP. Results from an experiment are represented in Fig. 1. Fig. 2 shows the simultaneous oxygen consumption.

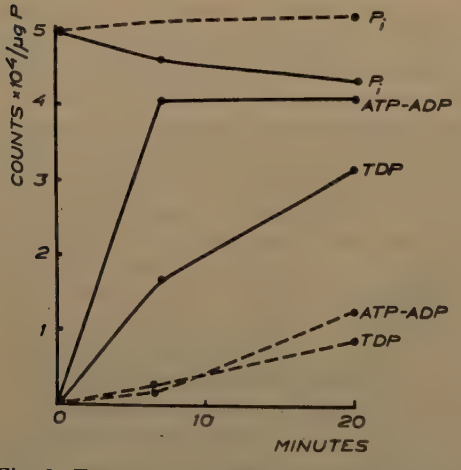


Fig. 1. Turnover rate of phosphate in TDP and ATP–ADP. For experimental details see text. ———, with substrate; — — —, without substrate.

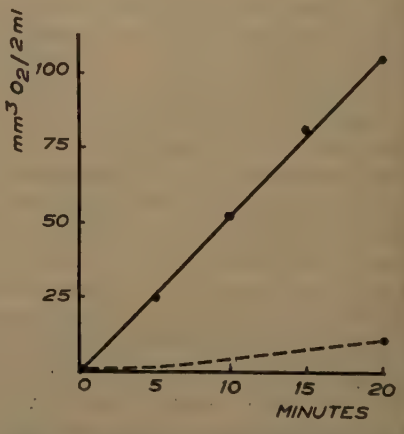


Fig. 2. Oxygen consumption with and without substrate. The measurements were performed in Warburg vessels with KOH in the center well and air in the gas space. For further details see text. ———, with substrate; — — —, without substrate.

Goethart (9) has shown that mitochondria from one gram of liver contain about 4 μg TDP. When isolating TDP from the mitochondria (each flask contained mitochondria from 6 gram of liver) 3 mg synthetic TDP was added to each sample. Hence the isolated TDP was a mixture of intramitochondrial and synthetic TDP, and the specific radioactivity of the intramitochondrial TDP is obtained by multiplying the specific radioactivity of the TDP mixture with a dilution factor.

The incorporation rate of P^{32} in βP of ATP is slower than in γP in liver mitochondria (Ernster, Ljunggren and Lindberg, 10; Whittam, Bartley and Weber, 11). In the presence of an excess of hexokinase the specific activity of βP approaches that of γP after 6 minutes (10).

From Fig. 1 it appears that the turnover rate of phosphate in the labile group of TDP is lower than that of ATP–ADP. With substrate present the radioactivity of the βP of TDP after 7 minutes is less than 50 per cent of that of the βP and the γP of ATP. After this interval the radioactivity of the labile phosphate groups of ATP may be considered to be the same (see 10, 11). After 20 minutes it is 75 per cent. Without substrate added the incorporation rate is low both in ATP and TDP.

Samples could only be taken at three intervals. Otherwise the amount of intramitochondrial TDP after the isolation procedure would have been too small to give an exactly measureable radioactivity. Different intervals were, however, tried in different experiments. The figures always fitted into curves of the type shown in Fig. 1, although the level varied slightly from one experiment to another.

The diagrams only intend to give an appreciation of the magnitude of the specific radioactivity of intramitochondrial TDP at different incubation intervals. Even if the radioactivity is high enough to give reliable values, the figures given by Goethart (4 μg of intramitochondrial TDP per gram of liver) may vary from species to species and from animal to animal. His figures are valid for rat liver, and the present experiments were made on guinea-pig liver. Errors also may occur at the extraction of TDP from the mitochondria. This is, however, not probable since the figures from different experiments give conformable values.

SUMMARY

The incorporation rate of radioactive phosphate in intramitochondrial TDP has been studied. The incorporation rate is dependent of the presence of pyruvate. With pyruvate as a substrate the incorporation rises to about $\frac{2}{5}$ of that of ATP–ADP after seven minutes.

ACKNOWLEDGEMENTS

I am indebted to Prof. P. E. Lindahl for valuable discussion, to Dr. Hans Löw for the supply of hexokinase, and to Mrs. Margot Aunver for technical assistance. Thanks are also due to Dr. A. L. Morrison, Messrs. Roche Products Ltd., for the gift of TDP. This work was made possible by grants from the *Swedish Natural Sciences Research Council.*

Institute of Zoophysiology, University of Uppsala, Uppsala, Sweden.

REFERENCES

1. KIESSLING, K.-H., Acta Chem. Scand., in press.
2. SCHNEIDER, W., and HOGEBOOM, G., J. Biol. Chem. *183* (1950), 123.
3. KIESSLING, K.-H., Acta Chem. Scand. *10* (1956), 1356.
4. SILIPRANDI, D., and SILIPRANDI, N., Biochim. Biophys. Acta *14* (1954), 52.
5. BERENBLUM, I., and CHAIN, F., Biochem. J. *32* (1938), 295.
6. BERGER, L., SLEIN, M., COLOWICK, S., and CORI, C., J. Gen. Physiol. *29* (1946), 379.
7. BAILEY, K., and WEBB, E., Biochem. J. *42* (1948), 60.
8. CARTER, H., Biochemical Preparations, Vol. 1 (1949), 5.
9. GOETHART, G., Biochim. Biophys. Acta *8* (1952), 479.
10. ERNSTER, L., LJUNGGREN, M., and LINDBERG, O., Acta Chem. Scand. *8* (1954), 658.
11. WHITTAM, R., BARTLEY, W., and WEBER, G., Biochem. J. *59* (1955), 590.

Tryckt den 12 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 49

Communicated 10 April 1957 by Arne Westgren and Karl Myrbäck

L- and D-Leucyl-glycine-splitting dipeptidases in *Elodea densa*

By Per Johan Palmcrantz

With 1 figure in the text

Studies on L-dipeptidases, splitting "natural" L-dipeptides, have been reviewed by several authors, e.g. by Greenberg and Winnick (1) and by Smith (2, 3, 4). Investigations on D-dipeptidases have been reviewed by Waldschmidt-Leitz (5). The various dipeptidases do not hydrolyze all dipeptides but are more or less specific for certain dipeptidic substrates. Crude enzyme preparations may be mixtures of different dipeptidases. From such mixtures certain specific dipeptidases have been isolated and characterized. The properties of different dipeptidase preparations show that L-dipeptidases and D-dipeptidases are distinct from each other; D-dipeptidases are less stable at dialysis, aseptic autolysis, unsuitable pH, higher temperatures etc. than the L-enzymes.

The effect of certain metal ions (Mg, Mn, Co, Zn etc.) on the activity of dipeptidases has been investigated extensively; different substrate-specific dipetidases are affected differently. Merten (6), e.g., working with D- and L-dipeptidases, found large variations in the activation or inhibition of the enzymes by different metal ions.

In most studies enzymes of animal origin have been used; plant dipeptidases are less well known. In the following some experiments of the leucyl-glycine splitting enzymes in leaves of the water-plant *Elodea densa* are reported. The plant was cultivated in an all-glass aquarium under constant conditions (7). Fresh material from the same culture was used in all experiments.

Enzyme preparations. Leaves were cut from the plant and were ground immediately in a porcelain mortar with quartz sand (purified by boiling with hydrochloric acid), glycerol (double-distilled, p.a.), Na_2HPO_4 (Sörensen, for enzyme investigations) and a few drops of toluene (quantities in the following). After grinding water was added and the suspension was centrifuged. The extracts were used after dilution in the activity determinations.

Substrates. D- or L-Leucyl-glycine, respectively. Both preparations (Hoffmann-La Roche, Basel) were of high purity; no free amino acids were present.

The determination of enzyme activity was carried out at 30° in glass-stoppered Erlenmeyer flasks immersed in a water thermostate. At suitable reaction times samples of the reaction mixtures were removed and boiled for 1 min to stop the reaction. In every reaction mixture pH was determined with a glass electrode.

The quantities of free amino acids formed were estimated by means of the ninhydrin method of Van Slyke, i.e., after rection of the amino acids with ninhydrin the evolved carbon dioxide was determined manometrically in the Van Slyke-Neill apparatus (Van Slyke *et al.* (8); modifica-

tions (9, 10, 11)). The reaction with ninhydrin was carried out in Thunberg tubes of essentially the same kind as those used by Hamilton and Van Slyke (10). A high-temperature stop-cock grease was prepared from aluminium distearate and paraffin oil as described in (10). With this grease the Thunberg tubes could be evacuated, heated to 100°C and kept for several days in a refrigerator without any air leaking in. Before addition of ninhydrin the test solutions were buffered with acid citrate mixture of pH 2.5 recommended by Van Slyke (8). After the carbon dioxide had been transferred to the Van Slyke-Neill apparatus the two-way stop-cock was sealed with mercury (MacFadyen (9)). The 0.5 *N* NaOH and the 2 *N* lactic acid were saturated with NaCl as recommended by the same author (9). Reabsorption of CO_2 while the meniscus in the apparatus is raised is then diminished, and the "i correction" which is applied because of the reabsorption of CO_2 will be smaller. The lactic acid solution contained hydrazine sulfate in order to prevent small errors due to volatile aldehydes (Hamilton (11)). After the addition of ninhydrin (Riedel- de Haën) and evacuation the Thunberg tubes were heated to 100°C for 10 min, cooled to room temperature, and the carbon dioxide was measured in the Van Slyke-Neill apparatus. Then the Thunberg tubes were heated a second time to 100°C for 10 min and the extra carbon dioxide evolved was determined. In this way a correction was obtained for a slow decomposition of protein during heating (9). Control analysis of L-histidine dihydrochloride gave the following results:

Calculated	51.3 μg amino-N
Found	
Volume of apparatus 0.5 ml	51.4; 50.8 μg
2.0 ml	51.9; 51.0 μg

Experiments with D-leucyl-glycine

Glycerol concentration. Leaves from 5 plants were mixed; a portion was used for dry weight determination. From other portions extracts were prepared using different amounts of glycerol. 2 ml extract, corresponding to 40 mg dry material, and containing 6.4 mg PO_4 was mixed with 2 ml substrate solution, containing 20 mg substrate. pH was brought to 8.4. Reaction time 24 h (Table 1). Higher activity was found at moderate glycerol concentration; at very high concentrations the activity decreased. Presence of glycerol does not seem to stabilize the enzyme.

Dependence on reaction time. At low degrees of hydrolysis the curve, degree of hydrolysis versus time, is a straight line (Table 2).

Dependence on pH. 2 ml enzyme solution, corresponding to 28 mg dry material and containing 0.6 ml glycerol and 16 mg PO_4 was mixed with 2 ml substrate solution. Small amounts of NaOH were added to the desired pH values (Table 3). The pH optimum seems to be about pH 8.5.

Table 1. Influence of glycerol concentration.

Glycerol concn. in react. mixture *M*	% Hydrolysis Immediately	Enzyme stored 20 days at 0°C	Decrease %
0	1.64	1.14	30
1.5	2.66	1.54	42
3.0	2.15	1.47	32

Table 2. 1.5 *M* glycerol in the reaction mixture.

Time (hours)	% Hydrolysis
0	0
2	0.047
13½	0.60
48	2.9

Table 3. Reaction time 48 h.

pH	% Hydrolysis
7.71	1.12
7.96	1.26
9.14	1.23
9.98	0.59

Table 4. Reaction time 24 h.

pH	Salt added	% Hydrolysis
7.46	None	1.24
7.41	$MgCl_2$, 9.7×10^{-3} *M*	1.26
8.39	None	2.58
8.42	$MgCl_2$, 9.7×10^{-5} *M*	2.14
8.32	$MgCl_2$, 9.7×10^{-3} *M*	0.95
8.40	$MnCl_2$, 1.00×10^{-4} *M*	2.17
7.70	$MnCl_2$, 1.00×10^{-2} *M*	1.03
8.08	None	1.06
7.81	$MgCl_2$, 9.7×10^{-5} *M*	1.02
8.06	$MnCl_2$, 1.00×10^{-4} *M*	1.05
7.78	None	0.59
8.05	$MgCl_2$, 9.7×10^{-4} *M*	0.63
7.93	$MnCl_2$, 1.00×10^{-3} *M*	0.44

Influence of Mg^{++} and Mn^{++} ions. 2 ml enzyme solution (corresponding to 22 mg dry material) containing 0.6 ml glycerol and 16 mg PO_4, 2 ml substrate solution, 1 ml salt solution ($MgCl_2$ or $MnCl_2$, p.a.), Table 4.

No obvious activation by Mg- or Mn-ions could be observed. At high concentrations of these ions the enzyme was inhibited.

Distribution of enzyme in different parts of the plant

The extracts (10 ml) contained 0.3 ml glycerol and 8 mg PO_4 per ml. For activity determination 2 ml extract and 2 ml substrate solution were mixed, reaction time 24 h (Table 5).

(*a*) Leaves and stem from 2 *Elodea* plants, about 30 cm long.
1. Leaves from upper 7 cm of stem.
2. Leaves from lower part of stem.
3. Stem.

(*b*) Leaves and stem from 3 *Elodea* plants.
1. Leaves from upper 3 cm of stem.
2. Leaves from lower part of stem.
3. Stem.

(*c*) 1. Leaves (young) from upper 3 cm of stem.
2. Leaves from stem between 3 and 10 cm from tip (mature leaves).
3. Leaves from lower part of stem (old leaves).

It appears from Table 5 that the enzyme activity in the stem is slight, compared to that of the leaves. Further it is clear that old leaves are less active than young. The highest activity is found in the mature leaves, 3–10 cm from the tip.

Table 5. Distribution of enzyme in different parts of the plant.

Series	Dry weight of material corresponding to 2 ml extract (*a*), g	% Hydrolysis (*b*)	*b*/*a*
*a*1	0.0193	0.725	37.5
2	0.076	0.975	12.9
3	0.048	0.234	4.9
*b*1	0.0268	1.18	44.1
2	0.0576	3.40	148
3	0.162	0.87	6.2
*c*1	0.0204	0.800	39.3
2	0.0232	1.27	55.0
3	0.0508	1.74	34.3

Experiments with L-leucyl-glycine

Dependence on pH. 1 ml extract corresponded to 12 mg dry material and contained 0.3 ml glycerol and 8 mg PO_4. Reaction time 24 h (Fig. 1). The experiments show that optimal activity is found at pH 9.0.

Influence of metal ions. 1 ml enzyme extract corresponded to 14 mg dry material and contained 8 mg PO_4. Reaction time 24 h. Table 6 shows that Mg- and Mn-ions exert a marked activating action on the enzyme. Optimum concentration seems to be about 1.5×10^{-3} *M* for both ions.

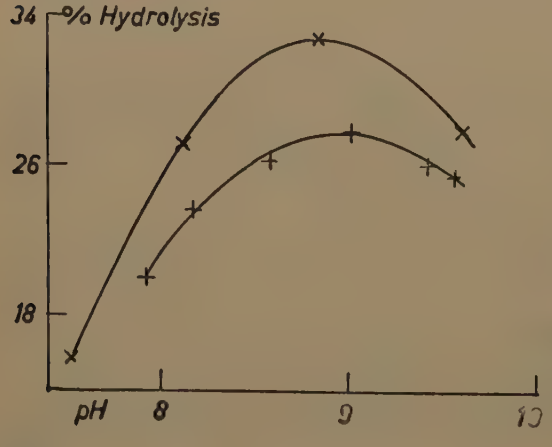


Fig. 1. Hydrolysis of L-leucyl-glycine: ×, fresh extract; +, extract stored 2 weeks at 0°C.

Table 6. Influence of Mg^{++} and Mn^{++} ions. Reaction time 24 h.

pH	Salt added	% Hydrolysis
9.4	None	44.1
9.4	$MgCl_2$, 9.7×10^{-4} *M*	50.3
9.4	$MgCl_2$, 19.4×10^{-4} *M*	50.0
9.4	$MgCl_2$, 29.1×10^{-4} *M*	48.6
9.4	$MgCl_2$, 38.8×10^{-4} *M*	47.0
8.8	$MgCl_2$, 97×10^{-4} *M*	34.1
9.2	None	34.9
9.3	$MnCl_2$, 2.0×10^{-4} *M*	38.6
9.2	$MnCl_2$, 5.0×10^{-4} *M*	39.3
9.2	$MnCl_2$, 10.0×10^{-4} *M*	41.0
8.9	$MnCl_2$, 40.0×10^{-4} *M*	38.9

Table 7. Reaction time 24 h.

Enzyme	pH	Concentration of added salt	% Hydrolysis
Extract after dialysis	9.0	None	18.3
	9.0	$MgCl_2$, 19.4×10^{-4} *M*	24.1
	8.9	$MgCl_2$, 9.7×10^{-4} *M* + $MnCl_2$, 10.0×10^{-4} *M*	22.2
	8.8	$MnCl_2$, 20.0×10^{-4} *M*	22.4
Original extract	9.3	None	28.1
	9.4	$MgCl_2$, 19.4×10^{-4} *M*	37.9

Table 8. Reaction time 24 h, pH 9.2.

Enzyme	Concentration of added salt	% Hydrolysis
Extract + $MgCl_2$ after dialysis	None	20.2
	$MgCl_2$, 19.4×10^{-4} *M*	20.9
Original extract	None	34.2
	$MgCl_2$, 19.4×10^{-4} *M*	39.4

Table 9. Reaction time 24 h, pH 9.0.

Enzyme	Concentration of added salt	% Hydrolysis
Extract after dialysis	None	2.76
	$MgCl_2$, 19.4×10^{-4} *M*	8.47
Original extract	None	36.2
	$MgCl_2$, 19.4×10^{-4} *M*	37.9

Dialysis experiments. Since it could be assumed that the crude enzyme extracts contain activating metal ions such as Mg^{++} or Mn^{++}, dialysis experiments were carried out to remove the activating ions. 15 ml extract was dialysed in a cellophane bag at 0°C against 3 l distilled water: the water was changed three times. After dialysis the volume of the enzyme solution was 27 ml, pH 7.6. Equal volumes of original extract and dialysed solution were used for activity determination (Table 7) at different concentrations of activating metal ions. It can be concluded from table 7, that activating metal ions, probably present in the extracts, have not been removed to any considerable extent by dialysis. In a second series a mixture of 15 ml enzyme extract and 2 ml 0.05 *M* $MgCl_2$ solution was dialysed as above. Volume after dialysis 32 ml, pH 7.6. Activity determinations with and without addition of $MgCl_2$ were carried out; parallel experiments with original enzyme extract (Table 8). The activity of the dialysed solution increases by only 3% in presence of added $MgCl_2$, whereas the activity of the original extract increases by about 15%. This shows that not even all added Mg ions have been removed by dialysis. Since this may possibly be due to the high pH of the solution dialysis experiments were carried out at lower pH values. However, the enzyme was found to be very unstable in acid solution. For instance, only 8% of the activity was left after storing for 40 h at 0°C and pH 5 (acetate buffer); after storing for 40 h at 0°C and pH 6 about 33% of the activity was found.

An enzyme extract was dialysed against 3×3 liter buffer solution of pH 6 for 24 h (Table 9). During dialysis the activity of the enzyme preparation decreased to about 16% of the original activity. However, in the presence of added Mg salt the activity increased by 210% whereas that of the undialysed extract increased by only 5%. This shows that dialysis at pH 6 removes most of the activating metal ions. At higher pH values the ions obviously are tightly bound by the enzyme and are not removed by dialysis.

The fact that the activating action of Mg- and Mn-ions is very different in the hydrolysis of D- and L-glycine, respectively, supports the conclusion in earlier investigations that the D- and L-forms of the peptide are hydrolysed by different enzymes.

Distribution of enzyme in different parts of the plant

The determinations were carried out as in the case of the D-peptidase (Table 10). It is seen that also the activity against the L-form of the peptide is by far highest in the mature leaves. These leaves are the richest in protein (12). In the young leaves, however, where the protein synthesis is more rapid, the peptidase content is lower. The experiments, therefore, tend to show that the leucyl-glycine splitting peptidases play no important role in protein synthesis.

Table 10. Distribution of enzyme in different parts of the plant.

Series	Dry weight (g) of material corresponding to 2 ml extract (*a*)	% Hydrolysis (*b*)	*b*/*a*
1	0.0183	11.9	650
2	0.0236	19.9	843
3	0.0688	36.7	533

SUMMARY

The hydrolysis of D- and L-leucyl-glycine by glycerol–water extracts of *Elodea densa* has been determined. The activity against the L-form was about 10 times that against the D-form. pH-Optimum of the D-peptidase was found to be 8.5, that of the L-peptidase 9.0. The activity of the D-enzyme did not increase on addition of Mg^{++} or Mn^{++} ions, but the L-enzyme was activated by these ions, optimum concentration about 1.5×10^{-3}. After dialysis of the extracts at pH 6, the activity increased very strongly on addition of Mg- or Mn-ions, showing that the crude extracts contained considerable amounts of activating metal ions. Dialysis at pH 7.6 was ineffective.

The activity of both peptidases was highest in mature leaves, lower in young and old leaves.

Institute of Organic Chemistry and Biochemistry, University of Stockholm.

REFERENCES

1. Greenberg, D. M., and Winnick, T., Ann. Rev. Biochem. *14*, 31 (1945).
2. Smith, E. L., Ann. Rev. Biochem. *18*, 35 (1949).
3. Smith, E. L., Advances in Enzymol. *12*, 191 (1951).
4. Smith, E. L., in Sumner, J. B., and Myrbäck, K., The Enzymes: Chemistry and Mechanism of Action, Academic Press Inc., New York, 1951, Vol. I, 793.
5. Waldschmidt-Leitz, E., Ergebn. Enzymforsch. *9*, 193 (1943).
6. Merten, R., Biochem. Z. *318*, 167 (1947).
7. Palmcrantz, P. J., Beitr. Biol. Pflanz. *29*, 220 (1952).
8. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., J. Biol. Chem. *141*, 627 (1941).
9. MacFadyen, D. A., J. Biol. Chem. *145*, 387 (1942).
10. Hamilton, P. B., and Van Slyke, D. D., J. Biol. Chem. *150*, 231 (1943).
11. Hamilton, P. B., and Van Slyke, D. D., J. Biol. Chem. *164*, 249 (1946).
12. Palmcrantz, P. J., Arkiv Kemi, In the press.

Tryckt den 12 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 50

Communicated 10 April 1957 by Arne Westgren and Karl Myrbäck

On the nitrogen metabolism in *Elodea densa*

By Per Johan Palmcrantz

With 8 figures in the text

Earlier investigations (cf. Chibnall (1)) have shown that the composition of the nitrogenous fractions of plant tissues changes with age of the tissue. For instance, Pearsall (2), investigating leaves of Swiss chard (*Beta vulgaris*, var. *cicla*), has found that the protein-N value that is high in young leaves, falls rapidly to a lower level as the leaves mature. It decreases only slightly in the mature leaves, but in old leaves there is again a rapid fall in protein nitrogen, accompanied finally by yellowing of the leaf and soon after by its death.

In the present investigation nitrogenous fractions in leaves of *Elodea densa* were studied. This plant is suitable for metabolic studies, since it can be grown at all seasons. Stem tips grow unlimitedly and non-rooted plants can be cultivated in an all-glass apparatus in nutrient solution under defined conditions (3).

Methods

Leaves from different parts of the stem of *Elodea* plants were collected and extracts were prepared. Different N-containing fractions were determined.

Preparation of extracts

A method similar to that of Vickery *et al.* (4) was used: The leaves were rinsed in distilled water and dried in an oven at 80°C for 1 h. Aqueous extracts were prepared by stirring the dried leaves with distilled water at 80°C for 10 min. The leaves were extracted three times in this way. The extracts were combined and centrifuged clear. Drying for 1 h at 80°C is sufficient; the very thin leaves dry up very quickly even at room temperature. The extraction of soluble nitrogenous substances is rather complete; in an experiment the amino acid content of extracts and residue was determined with ninhydrin:

	μg N
First extract (2 ml) + second extract (2 ml)	159.4
Third extract (2 ml)	9.7
Residue (leaves) corresponding to 2 ml extract	8.5

Analytical methods

Total N was estimated according to the technique described by Miller and Miller (4). However, in the case of *Elodea* material direct nesslerisation is not feasible, because of an opalescence due to calcium and magnesium salts. The following modification had to be adopted: After combustion

ammonia was distilled over in a micro Kjeldahl apparatus and taken up in the same quantity of sulfuric acid as that used in the combustion. When the Nessler reagent was added after dilution completly clear solutions were obtained. A standard curve was obtained with known quantities of ammonium sulfate.

Amino acid N was determined in the Van Slyke-Neill apparatus according to a modification of the ninhydrin method described earlier (5).

Amino-N was determined in the Van Slyke apparatus using the nitrite method.

Asparagine- and glutamine-N was determined by vacuum distillation of ammonia, in principle according to the methods of Pucher *et al.* (6) and Vickery *et al.* (7), but smaller volumes were used; volume of distillation flask 50 ml, volume at nesslerisation 3.6 ml.

When known quantities of ammonia were determined in the micro distillation apparatus used by Pucher and others the yield was not quantitative. It was found, however, that a quantitative yield could be obtained, provided that the following precautions were taken:

A glass filter is placed before the wash-bottle to prevent dust etc. to enter the apparatus. The wash-bottle is immersed in a water-bath of 54–56°C and the connection between wash-bottle and distilling flask is insulated with asbestos. The gas stream must be rather brisk. The distillate is taken up in diluted sulfuric acid and poured through a glass filter into a Kjeldahl flask, previously treated with boiling sulfuric acid. A small piece of quartz (not glass) is added to prevent bumping and the distillate is evaporated (protected against dust) to a very small volume. 3 ml water and 0.6 ml Nessler reagent are added. It was found necessary to use twice redistilled water throughout (once with sulfuric acid and permanganate, once with NaOH and permanganate, all reagents p.a.).

Extinction was determined in a Leitz photometer. A standard curve was obtained with known amounts of pure ammonium sulfate.

Experiments

The following series of determinations on *Elodea* leaf material were carried out:

Series 1.—Leaves from *Elodea* plants rooted in soil, grown in daylight (Botanical Institute, University). Ten series in the course of one year (25/9, 12/10, 20/11 1948, 17/1, 1/3, 8/4, 16/5, 16/6, 2/8, 12/9 1949). In each series ten samples were taken with 12 leaves in each sample. Dry weight and total N (Kjeldahl) in extracts and insoluble residues were determined. In two series (4/10 and 20/10 1948) four and five samples, respectively, with 70 leaves in each sample were taken. Dry weight, amino-N in extract and total N in insoluble residue were determined.

Series 2.—Leaves from plants grown in nutrient solution under constant conditions (5); five series (25/5, 11/10, 30/10, 15/11 and 30/11 1951), 4 samples in each series, 50 leaves in each sample. Dry weight and total N, amino acid N, amide- + ammonia N, and glutamine- + ammonia N in the extracts were determined; total N was determined in the insoluble residues.

The experiments demonstrate the variation of dry weight and the different N-fractions with the development of the leaves. The changes are due to maturating and aging of the leaves, but also to interaction of different parts of the plant, e.g. young growing parts receiving substances from older tissues, cf. Pearsall (2). The growth of the *Elodea* plants was rather steady and it could be assumed that all leaves pass through nearly the same stages of development.

Results

SERIES 1.—The curves in Figs. 1–3 refer to material from this series. They show the variations of dry weight, non-extractible and extractible N with the distance of the leaves from the stem tip, i.e. the variations with age of the leaves. The curves representing dry weight and non-extractible N are very similar to each other; they in-

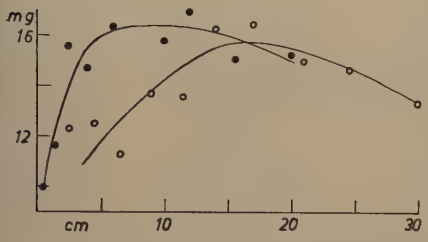


Fig. 1. Dry weight of 12 leaves at different distances from the stem tip (cm): ●, 25.9.1948; ○, 12.10.1948.

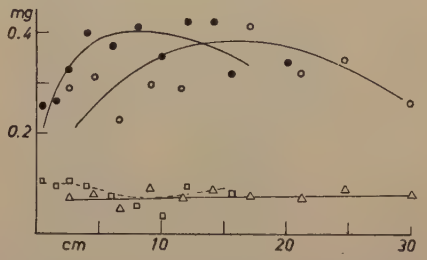


Fig. 2. Nitrogen distribution in leaves (12 in each sample) at different distances from the stem tip (cm): ●, non-extractible N, 25.9.1948; ○, non-extractible N, 12.10.1948; □, extractible N, 25.9 1948; △, extractible N, 12.10.1948.

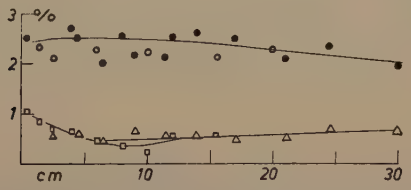


Fig. 3. Non-extractible N in per cent of dry weight: ○, 25.9.1948; ●, 12.10.1948. Extractible N in per cent of dry weight: □, 25.9.1948; △, 12.10.1948.

crease to a maximum and then decrease. The values of non-extractible N in per cent of dry weight are nearly constant in these experiments. Thus this fraction, probably containing chiefly proteins, represents a rather constant part of the leaf dry weight during most of their development. Extractible N, i.e. chiefly non-protein N, is rather constant. Extractible N in per cent of dry weight is highest near the stem tip (young leaves in which synthesis of proteins overweighs) and at the lowest part of the stem (old leaves where protein degradation overweighs).

In spite of the great differences in the life-conditions of the plants used in this series of determinations, the curves for different times of the year are very similar to each other and the various N-containing fractions did not vary considerably.

Fig. 4. demonstrates the changes of amino-N of the extracts. The amino-N values in per cent of dry weight is highest near the stem tip and at the lowest part of the stem.

SERIES 2.—In this series aliquots of the extracts were used for determination of various nitrogenous fractions.

Table 1. Data for 12 mature leaves, approximately at dry weight maximum.

Date	Dry weight mg	Non-extractible N		Extractible N	
		mg	% of dry weight	mg	% of dry weight
$^{25}/_{9}$	16.0–16.5	0.40–0.42	2.30–2.70	0.060–0.100	0.35–0.70
$^{12}/_{10}$	15.5–16.0	0.38–0.40	2.30–2.60	0.070–0.090	0.45–0.60
$^{20}/_{11}$	10.6–11.1	0.34–0.37	3.30–3.50	0.045–0.065	0.45–0.60
$^{17}/_{1}$	9–12	0.33–0.41	3.50–3.90	0.060–0.090	0.60–0.85
$^{1}/_{3}$	7.2–7.6	0.24–0.26	3.20–3.30	0.055–0.070	0.60–0.80
$^{8}/_{4}$	7.7–8.2	0.28–0.31	3.50–3.70	0.055–0.070	0.65–0.85
$^{16}/_{5}$	10.2–10.5	0.34–0.36	3.00–3.60	0.040–0.060	0.40–0.60
$^{16}/_{6}$	6.4–10.5	0.22–0.35	3.20–3.50	0.030–0.040	0.35–0.45
$^{2}/_{8}$	7.5–8.7	0.25–0.32	3.50–4.10	0.035–0.050	0.40–0.85
$^{12}/_{9}$	7.5–8.1	0.19–0.21	2.60–3.00	0.030–0.050	0.40–0.60

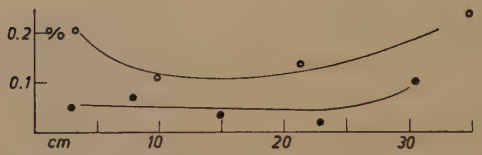


Fig. 4. Amino-N (van Slyke) in leaf extract (per cent of dry weight): ○, 4.10.1948; ●, 20.10 1948. Leaves at different distances from the stem tip (cm).

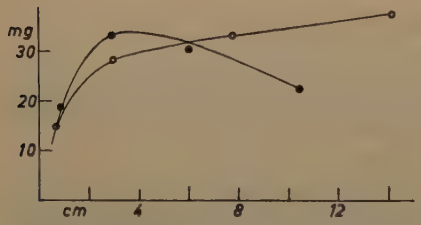


Fig. 5. Dry weight of 50 leaves at different distances from the stem tip: ●, 15.11. 1951; ○, 30.11.1951.

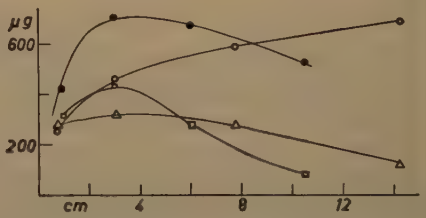


Fig. 6. Nitrogen distribution in leaves (50 in each sample) at different distances from the stem tip (cm): ●, non-extractible N, 15.11.1951; ○, non-extractible N, 30.11. 1951; □, extractible N, 15.11.1951; △, extractible N, 30.11.1951.

The curves (Figs. 5–8) showing dry weight and non-extractible N are similar to the corresponding curves found in Series 1. The curves for soluble N, amino acid N, and amide- + ammonia N have all pronounced maxima near to the stem tip. In all determinations of glutamine- + ammonia N and in the determinations of amide- + ammonia N in extracts from leaves from the lower part of the stem the quantities of ammonia were to small to allow accurate determination (the nesslerisation was sometimes disturbed by precipitation of red crystals; cf. (4).) It can be concluded that in

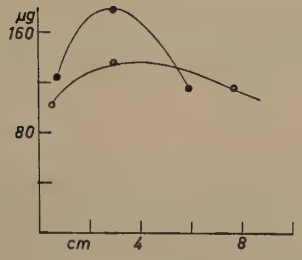


Fig. 7. Amino acid α-N in extracts of 50 leaves at different distances from the stem tip (cm): ●, 15.11.1951; ○, 30.11. 1951.

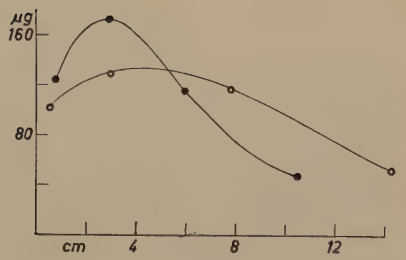


Fig. 8. Amide- + ammonia N in extracts of 50 leaves at different distances from the stem tip (cm): ●, 15.11.1951; ○, 30.11.1951.

young leaves the amide-N fraction, which is rather large, consists almost entirely of asparagine. The curves for amino acid N practically coincide with the curves for amide- + ammonia N; it seems therefore that the amino acids fraction contains chiefly asparagine. Other free amino acids can be present only in very small quantities. Since asparagine, in the Kjeldahl determination, yields twice the amount of N calculated from the determinations of amino acid N and amide-N, it can be seen that, although most of the total nitrogen, at least in younger leaves, originates from asparagine, there are also other nitrogenous compounds present. These compounds were most abundant near the stem tip, and it seems probable that they play a role in protein synthesis in the young leaves. Earlier investigations (cf. (1)) have shown, that the large amounts of asparagine, found in stem tips, are utilized for protein synthesis.

SUMMARY

Certain fractions of N-containing substances have been determined in leaves from different parts of the stem of *Elodea densa*. With material from plants, rooted in soil and grown in daylight, it was found that dry weight and quantity of non-extractible N (protein) were highest in mature, fully outgrown leaves. The values of non-extractible N in per cent of dry weight were practically constant. Total extractible N and total amino-N in the extracts were nearly constant. Total extractible N and total amino-N in the extracts in per cent of dry weight were highest near the stem tip, where protein synthesis overweighs, and at the lowest part of the stem, where protein degradation overweighs.

With material from plants grown in nutrient solution under constant conditions the same results were obtained concerning dry weight and non-extractible N. The values of extractible N, amino acid N and amide-N had pronounced maxima near the stem tip. The amide fraction contained chiefly asparagine; only traces of glutamine and ammonia were present. Most of the amino acid N, as determined by the ninhydrin method, originates from asparagine.

Institute of Organic Chemistry and Biochemistry, University, Stockholm.

REFERENCES

1. CHIBNALL, A. C., Protein Metabolism in the Plant. New Haven, Conn., 1939.
2. PEARSALL, W. H., Endeavour *8*, 99 (1949).
3. PALMCRANTZ, P. J., Beitr. Biol. Pflanz. *29*, 220 (1952).
4. MILLER, G. L., and MILLER, E. E., Anal. Chem. *20*, 481 (1948).
5. PALMCRANTZ, P. J., Arkiv Kemi. In the press.
6. PUCHER, G. W., VICKERY, H. B., and LEAVENWORTH, C. S., Ind. and Eng. Chem., Anal. Ed. *7*, 152 (1935).
7. VICKERY, H. B., PUCHER, G. W., CLARK, H. E., CHIBNALL, A. C., and WESTALL, R. G., Biochem. J. London *29*, 2710 (1935).

Tryckt den 12 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 51

Communicated 5 June 1957 by Per Eric Lindahl

The effect of the metabolization of different substrates upon the incorporation of P^{32} into mitochondrial thiamine diphosphate[1]

By Karl-Heinz Kiessling

In a previous paper (1) it has been shown that radioactive inorganic phosphate was incorporated into TDP in mitochondria, when pyruvate was metabolized. Without pyruvate the incorporation was comparatively low. Synthetic TDP added to the mitochondria did not exchange P^{32} to the same degree. Nor did labelled synthetic thiamine phosphates act as phosphate donors as had been found by Ogata *et al.* (2) with a liver cyclophorase system.

This communication deals with the influence of three different substrates upon the incorporation of P^{32} into intramitochondrial TDP. Mitochondria from guinea pig liver have been incubated with substrate, inorganic radioactive phosphate, ADP, hexokinase, and glucose. The substrates used were pyruvate, α-ketoglutarate, and succinate, and the experimental conditions as well as the methods for isolating TDP and ATP-ADP were the same as in previous investigations on liver mitochondria from guinea pig (1). The incubation time was 25 minutes. Results from an experiment are shown in Table 1.

Table 1.

Substrate	TDP counts/μg hydrolyzable P	ATP-ADP counts/μg hydrolyzable P	P_i counts/μg P	Oxygen consumption mm^3/25 min.
Pyruvate	1.11×10^5	1.61×10^5	1.72×10^5	113
α-ketoglutarate	0.37×10^5	1.23×10^5	1.75×10^5	167
Succinate	0.85×10^5	1.37×10^5	1.57×10^5	175
None	0.15×10^5	0.28×10^5	1.77×10^5	19

It appears from the table that the incorporation of radioactive inorganic phosphate into TDP is more rapid with pyruvate and succinate than with α-ketoglutarate.

These results are only partly in agreement with those of Ogata *et al.* (2). These authors used P^{32}-labelled TDP added to a liver cyclophorase system, and examined to what extent P^{32} was transferred to the adenylic acid system, when

[1] *Abbreviations:* TDP, thiamine diphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; P^{32}, radioactive phosphate.

pyruvate, α-ketoglutarate, or succinate were metabolized. The highest radioactivity in ATP was found with pyruvate and α-ketoglutarate as substrates.

This work is part of investigations supported by the Swedish Natural Science Research Council.

Institute of Zoophysiology, Uppsala, Sweden.

REFERENCES

1. KIESSLING, K.-H., Acta Chem. Scand., in press.
2. OGATA, K., NOHARA, H., MORITA, T., and KAWAI, K., J. Biochem. *42*, 13 (1955).

Tryckt den 12 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 52

Read 24 April 1957

Inhibition of yeast invertase (saccharase) by metal ions

V. Inhibition by mercury compounds

By Karl Myrbäck

With 5 figures in the text

Yeast invertase is strongly inhibited by extremely small concentrations of mercury (II) chloride. The inhibition can be reversed quantitatively, e.g. by hydrogen sulphide, provided that enzyme and inhibitor have not been incubated together for any considerable time. The inhibition is competitive, i.e. the substrate (saccharose) has a protective action (1, 2, 3). In the optimal pH range of the enzyme (pH 3.5–5.5) the inhibition does not vary appreciably with pH; above pH 5.5 the inhibition may increase somewhat (3). The relative activity (RA) of a highly purified yeast invertase preparation (If = 150) in the presence of various concentrations of $HgCl_2$ at pH 5.3 is shown in Fig. 1. Mercury(I) nitrate has the same or possibly even a stronger inhibitory power than $HgCl_2$ (Fig. 1).

Experiments with $HgCl_2$

For a comparison with the results mentioned above, inhibition experiments were carried out with an enzyme preparation, partly purified from yeast extract, of much lower specific acitivity (If = 2) and with different times of incubation of enzyme and inhibitor:

(*a*) *Experiments without incubation of enzyme and inhibitor.*—10 ml acetate buffer of pH 5.3 (*M* sodium acetate + acetic acid), 4.75 g saccharose, solution of $HgCl_2$ and water to 99 ml. After temperature equilibration at 30°, 1 ml enzyme solution was added and the relative activity (RA) of the enzyme was determined as described earlier (4).

(*b*) *Incubation experiments.*—10 ml acetate buffer of pH 5.3, solution of $HgCl_2$ and water to 79 ml. After temperature equilibration at 30°, 1 ml enzyme solution was added. The mixture was kept at 30° for a given time after which 20 ml of a solution (30°) containing 4.75 g saccharose was added and the relative activity of the enzyme was determined.

The results are shown in Table 1. For a comparison with the experiments mentioned above, the RA values without incubation are plotted in Fig. 1 (△). It appears that the two invertase solutions with widely different specific activities are inhibited to practically the same degree by various concentrations of $HgCl_2$. It is concluded that the impurities in the less pure enzyme preparation do not bind appreciable amounts of mercury.

Table 1. Inhibition by $HgCl_2$ at pH 5.3.

Inhibitor concentration is given as mole/l in the complete reaction mixture.

$HgCl_2$ mole/l	*Relative activity (RA)* Incubation time at 30°, min.		
	0	30	120
10^{-7}	97.4	63.7	57.7
2.5×10^{-7}	70.5	29.6	29.6
5×10^{-7}	52.2	28.2	28.2
10^{-6}	40.0	30.5	30.5
	37.3	31.8	31.8
2.5×10^{-6}	35.5	30.5	30.5
	30.9	30.9	27.3
5×10^{-6}	30.9	25.0	22.7

The RA values with and without incubation of enzyme with the inhibitor are illustrated by Fig. 2. It appears that at $HgCl_2$ concentrations $<10^{-6}$ *M* the inhibition is strongly dependent on the incubation time. At higher inhibitor concentrations the influence of the incubation time is small or none. Furthermore it is clearly seen from Fig. 2 that, in the concentration range investigated, on increase of inhibitor concentration and increase of incubation time, a limit of inhibition is reached at a relative activity of 25–30 (dashed curve). At further increase of the Hg concentration the RA values seem to decrease very slowly. The explanation would be that Hg reacts with more than one group (or more than one type of groups) of the enzyme molecule. A certain number of groups in the enzyme protein appear to have a very high affinity for Hg and, consequently, give Hg complexes at very low concentrations of $HgCl_2$. These complexes, however, are not quite inactive but have a relative activity of 25–30. At considerably higher inhibitor concentrations further groups of the enzyme molecule may react with Hg to yield complexes of lower relative activity, finally possibly inactive complexes.

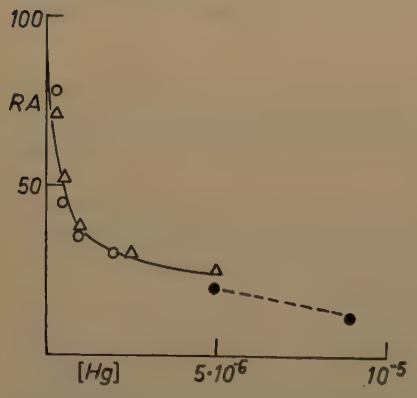


Fig. 1. Inhibition of yeast invertase (If = 150) by $HgCl_2$ (○) and by Hg_2Cl_2 (●); inhibition of enzyme preparation of lower specific activity (If ~ 2) by $HgCl_2$ (△).

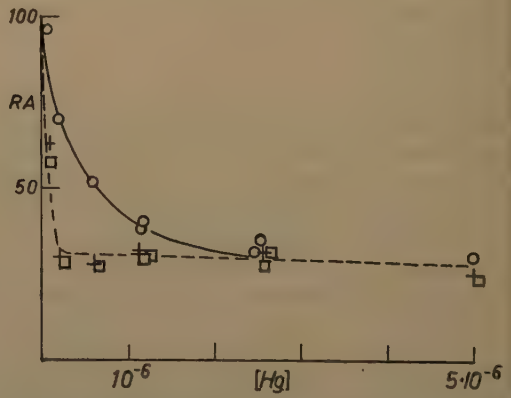


Fig. 2. Inhibition of invertase by $HgCl_2$: (*a*) without incubation of enzyme with the inhibitor (○), (*b*) with 30 min. incubation (+), (*c*) with 120 min. incubation (□).

Table 2. Final concentration of $HgCl_2$ in reaction mixture 10^{-6} *M*.

Concentrations of histidine and imidazole are the final concentrations in the assay mixtures.

Concentration of protecting substance	*Relative activity (RA)* Incubation time, min. 0	30	120
—	38.1		
Histidine, 4×10^{-3} *M*	58.7	66.8	59.1
1.6×10^{-2} *M*	—	59.1	67.8
Imidazole, 10^{-2} *M*	45.5		

In earlier papers of this series (5, 6) it has been shown that the inactivation of yeast invertase by silver ions is reversed by histidine in a way that may suggest that imidazole groups of the enzyme are involved in the combination of the enzyme with silver ions. For a comparison the protecting action of histidine and imidazole against inactivation by $HgCl_2$ was investigated:

(*a*) *Experiments without incubation of $HgCl_2$ and protecting substance.*—Two solutions were prepared: (1) 10 ml acetate buffer of pH 5.3, 4.75 g saccharose, solution of $HgCl_2$ and water to 50 ml; (2) solution of protecting substance, 1 ml enzyme solution and water to 50 ml. At 30° these solutions were mixed and the RA was determined.

(*b*) *Experiments with incubation of protecting substance and $HgCl_2$.*—10 ml acetate buffer of pH 5.3, solution of $HgCl_2$, solution of protecting substance and water to 99 ml. After incubation at 30° 1 ml enzyme solution was added and the RA was determined.

The results of these experiments (Table 2) indicate that histidine and imidazole have a rather weak protecting action. It has been concluded earlier (3) that Hg is not bound to the same group(s) of the enzyme protein as is the silver ion, and this conclusion seems to be supported by the above experiments on the protecting action of histidine.

Reactivation experiments on the Hg-inactivated yeast invertase were also carried out using a sulphydryl compound, viz. reduced glutathione (GSH). Results are recorded in Table 3. This table shows that the enzyme inactivation by $HgCl_2$, even after incubation of enzyme and inhibitor for 2 h, is reversed to a very considerable extent. However, the reactivation is a very slow process.

Table 3. Final concentration of $HgCl_2$ 10^{-6} *M*; final concentration of GSH 10^{-5} *M*. pH 5.3.

Composition and treatment	Relative activity
Enzyme + $HgCl_2$, incubation 30 min. at 30°	25.5
Enzyme, $HgCl_2$ and GSH added at the same time	100
Enzyme + $HgCl_2$, 30 min.; GSH and substrate added	27.3
Enzyme + $HgCl_2$, 30 min.; GSH, 120 min.; substrate added	45.5
Enzyme + $HgCl_2$, 120 min.; GSH, 240 min.; substrate added	65.5
Enzyme + $HgCl_2$, 120 min.; GSH, 17 h; substrate added	80.0
Enzyme + $HgCl_2$, 120 min.; GSH, 43 h.; substrate added	85.5

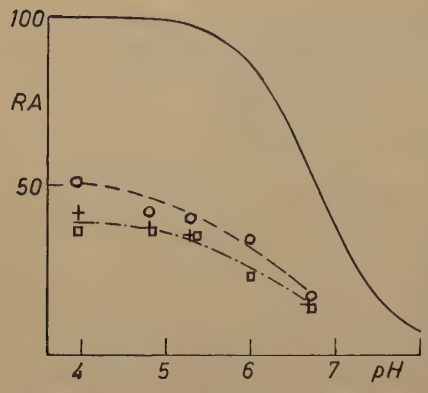


Fig. 3. Dependence of inhibition by 10^{-6} *M* $HgCl_2$ upon pH: ○, no incubation; +, 30 min. incubation; □, 120 min. incubation. The full-drawn curve is the activity–pH curve of the enzyme without inhibitor.

Experiments on the inhibition by $HgCl_2$ at different pH values were carried out in acetate buffers; the buffer concentration has no marked influence on the degree of inhibition. The results are illustrated by Fig. 3. In agreement with earlier results (3) it is found that the inhibition, with or without incubation of the enzyme together with the inhibitor, is rather independent of pH in the range investigated. This means that Hg is bound by groups of the enzyme molecule that do not change their charge appreciably in the pH range in question. Without any assumptions at this stage concerning the nature of the Hg-binding groups of the enzyme it may be pointed out that free SH groups of proteins generally have p*K* values well outside the pH range investigated here.

Experiments with organomercury compounds

Phenylmercury acetate (Ph-HgAc). Experiments with various concentrations of the inhibitor were performed at pH 5.3 as described for $HgCl_2$ (Table 4). It is seen from this table that the inhibitions at the lowest concentrations of Ph-HgAc, $< 10^{-6}$ *M*,

Table 4. Inhibition by phenylmercury acetate.

Inhibitor concentration mole/l	*Relative activity* (*RA*) Incubation time, min. 0	30	120
5×10^{-8}	100	—	—
10^{-7}	86.4	79.5	79.5
2.5×10^{-7}	60.9	40.9	40.9
	73.8		
5×10^{-7}	45.5	36.4	36.4
	63.6	40.9	40.9
10^{-6}	30.4	21.8	20.0
	25.0		
2.5×10^{-6}	21.4	20.9	20.9
	19.5		
10^{-5}	16.8	16.8	
5×10^{-5}	11.4	11.4	

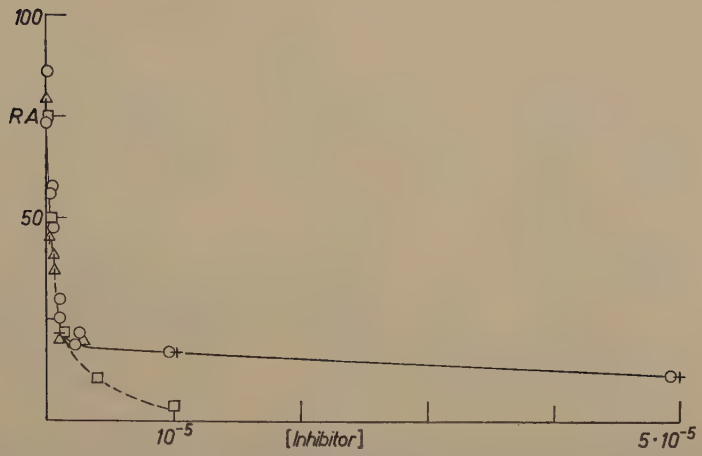


Fig. 4. Relative activity (RA) of invertase at different concentrations of Ph-HgAc: O, no incubation; +, 30 min.; △, 120 min. incubation. $AgNO_3$: □.

increase with incubation time, but constant values are reached in less than 30 min. At higher concentrations of Ph-HgAc the inhibition is practically independent of incubation time. Fig. 4 shows that the relative activity of the enzyme in the presence of increasing concentrations of the inhibitor falls to RA $\sim$ 20 at a concentration $\sim 2.5 \times 10^{-6}$ *M*. Further increase of the Ph-HgAc concentration causes only a very slow decrease of the RA. Even at a more than 20-fold concentration of the inhibitor the RA is still about 11. Obviously the inactivation of yeast invertase by Ph-HgAc, like the inhibition by $HgCl_2$, involves the formation of at least two different complexes: One is formed with a very high affinity, i.e. at very low inhibitor concentrations; this complex seems to have a relative activity $\sim$20. At higher inhibitor concentrations more Ph-HgAc is bound by the enzyme to form one or more further complexes with RA $<$ 20. This supports the assumption, probable *per se*, that $HgCl_2$ and Ph-HgAc react with the same groups of the enzyme molecule.

The dependence of the inactivation by Ph-HgAc upon pH was determined; inhibitor concentration 5×10^{-7} *M* and different incubation times. It appears from Fig. 5 that the inhibition–pH curves have the same general shape as the corresponding curves for $HgCl_2$ (Fig. 3).

Experiments were also carried out with a constant inhibitor concentration, 5×10^{-7} *M*, and three different substrate concentrations (Table 5). It appears that at least the initial rapid inactivation is competitive; as mentioned above the same is found in the case of the inhibition by $HgCl_2$.

The action of histidine as a protecting agent against the inactivation by Ph-HgAc was investigated: Mixtures of inhibitor solution, acetate buffer of pH 5.3, enzyme and histidine solution (80 ml) were incubated at 30°. 4.75 g saccharose in 20 ml were then added and the relative activity was determined (Table 6). Histidine has unmistakably a protecting action, but only at fairly high concentrations, i.e. the affinity of histidine for the inhibitor is very low compared to that of the enzyme.

p-Chloromercury benzoate (*p*-HOCO-Ph-HgCl): the preparation used was from the Amend Drug & Chemical Co., New York; *m-Chloromercury benzoate* (*m*-HOCO-Ph-HgCl) and *o-Hydroxymethyl-phenylmercury chloride* (*o*-$HOCH_2$-Ph-HgCl): samples of

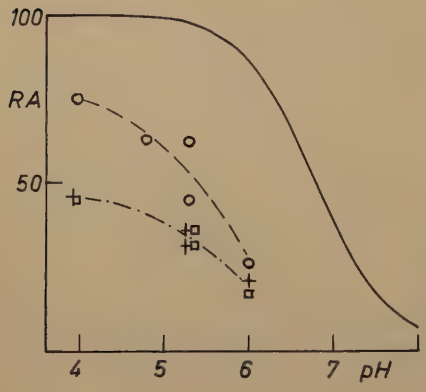


Fig. 5. Dependence of inactivation by 5×10^{-7} Ph-HgAc upon pH: O, no incubation; +, 30 min. incubation; □, 120 min. incubation. The full-drawn curve is the activity–pH curve of the non-inhibited enzyme.

these substances were a gift from Dr. Kurt Torssell of this laboratory who had prepared them via the corresponding substituted phenylboric acids. Inhibition experiments with the three substituted phenylmercury chlorides were carried out as described above (Table 7). The table shows that the three mercury compounds are potent inhibitors. At very small inhibitor concentrations the degree of inhibition depends on the time of incubation of enzyme with the inhibitors. At higher inhibitor concentration the effect of incubation is insignificant or non-existing. The *p*- and *m*-chloromercury-benzoates have practically the same inhibitory effect on invertase; *o*-hydroxymethylphenyl-mercury chloride, however, is a much more potent inhibitor.[1] The concentration of the latter compound causing immediate inhibition to 50% is $\sim 1.3 \times 10^{-6}$ whereas that of the first two compounds is $\sim 7 \times 10^{-6}$.

As in the case of $HgCl_2$ and Ph-HgAc it seems clear that the inhibition of yeast invertase by the three substituted phenylmercury chlorides is a reaction with (at least) two phases. Moderate concentrations of the inhibitors cause the formation of enzyme-inhibitor compounds with a relative activity around 20 (not necessarily the same for all mercury inhibitors). In the case of $HgCl_2$, Ph-HgAc and *o*-$HOCH_2$-Ph-HgCl this stage is reached at an inhibitor concentration $\sim 5 \times 10^{-6}$ *M* without any incubation of enzyme and inhibitor. After incubation for 1–2 h the same stage is reached at an

Table 5. Inactivation by phenylmercury acetate at different substrate concentrations. pH 5.3.

Substrate concentration mole/l	*Relative activity* Incubation, min. 0	120
0.139	63.6	40.9
0.278	79.6	41.3
0.556	94.8	47.3

[1] It may be of interest to mention in this connection that of the corresponding substituted phenylboric acids the *o*-hydroxymethylphenylboric acid is much more toxic to higher plants (7) and to certain bacteria (8) than are the *p*- and *m*-carboxyphenylboric acids.

Table 6. Final inhibitor concentration in assay mixture. 5×10^{-7} *M*.

Concentration of histidine mole/l	Incubation time min.	Relative activity
0	—	45.5
4×10^{-4}	30	68.2
	120	68.2
2×10^{-3}	0	68.1
	30	69.6
	120	81.5
4×10^{-3}	30	84.5
	120	84.5

Table 7. pH 5.3. Inhibitor concentration is that of the complete reaction mixture.

Inhibitor mole/l	Incubation time	Relative activity		
		p-HOCO-Ph-HgCl	*m*-HOCO-Ph-HgCl	*o*-$HOCH_2$-Ph-HgCl
5×10^{-7}	0			75.7
10^{-6}	0			55.1
	120 min.			30.4
2×10^{-6}	0	80.0	79.5	
	30 min.	55.2	69.9	
	90 min.	40.4		
	16 h	29.4		
2.5×10^{-6}	0			31.2
5×10^{-6}	0	58.1	58.1	22.8
	30 min.			22.8
10^{-5}	0	44.1	36.7	19.1
2×10^{-5}	0		27.2	
10^{-4}	0	20.2	14.6	

inhibitor concentration $\sim 10^{-6}$ *M*. *p*- and *m*-HOCO-Ph-HgCl have, immediately, a considerably weaker action, but also in these cases incubation of enzyme with small amounts of the inhibitors lowers the relative activity to ~ 20.

Increase of the concentration of the mercury inhibitors in the assay mixtures to 10^{-5} or even 10^{-4} *M* decreases the relative activity only slightly. Probably the complexes with RA ~ 20 add more inhibitor molecules and are transformed into complexes with lower RA.

Discussion

The inhibitors tested towards yeast invertase in the experiments described here are generally regarded as rather specific for sulphydryl groups of proteins. Most authors seem to agree, however, that inhibition of an enzyme by *p*-chloromercurybenzoate, $HgCl_2$, etc., cannot be taken as proof of the sulphydryl nature of the enzyme (9, 10). In the present case the strong inhibition of the enzyme by $HgCl_2$, Hg_2Cl_2

and the organomercury compounds, and the reversibility of the inhibition by SH compounds (H_2S, reduced glutathione) may well be explained as due to the formation of Hg mercaptides. The weak influence of pH on the inbibition is in agreement with this explanation. The competitive nature of the first phase of the Hg inhibition would then lead to the further conclusion that SH groups are involved in the combination enzyme–substrate. It appears necessary, however, at this point to compare the inhibition of yeast invertase by the Hg compounds with the inhibition of the enzyme by silver ions.

It seems to be assumed generally that the silver ions (and several other heavy metal ions) inhibit certain enzymes in the same way as the mercury compounds, i.e. by combination with sulphydryl groups of the enzyme proteins. However, a comparison of the inhibition of yeast invertase by Ag^+ and that by the mercury compounds reveals several characteristic dissimilarities. The Ag inhibition is non-competitive (3), the Hg inhibition is competitive; the Hg inhibition shows no distinct dependence on pH, whereas the Ag inhibition depends strongly on pH, indicating a reaction between the Ag ion and a group of the enzyme protein with pK 6–7; the complex formation of Ag^+ with low molecular SH compounds (reduced glutathione, thioglycolic acid) is not dependent on pH in the range 4–7 (11) and the same is found for the combination of Ag^+ and methionine. As pointed out above, the inhibition of yeast invertase with Hg compounds leads to complexes with a certain residual activity, whereas the enzyme-Ag compound is inactive (cf. the dashed curve (□) in Fig. 4.).

All these facts seem to exclude the assumption that, in the case of yeast invertase, Ag and Hg are bound by the same group(s) of the enzyme protein. If the Hg compounds are supposed to react with SH groups—and as far as our experience goes, nothing seems to contradict this assumption—it appears necessary to assume that Ag^+ is bound by non-SH groups or by SH groups having properties strongly deviating from the normal. As pointed out in earlier papers (5, 6) participation of histidine residues of the enzyme in the combination with Ag seems a reasonable assumption.

SUMMARY

Yeast invertase is strongly inhibited by $HgCl_2$, phenylmercury acetate, *p*- and *m*- chloromercury-benzoates and *o*-hydroxymethylphenylmercury chloride. The inhibition of the enzyme by these substances is a reaction of at least two phases. The first phase yields complexes, retaining about 20 % of the original activity of the enzyme. This phase of the inactivation reaction is competitive. The inhibition by the Hg compounds is not distinctly dependent on pH in the range 4–7. In this and several other respects the Hg inhibition distinguishes itself from the inactivation of the enzyme by Ag^+. The probable nature of the metal-binding groups of the enzyme protein has been disussed.

ACKNOWLEDGMENT

Thanks are due to Professor O. Lindberg and Dr. Kurt Torssell for samples of organomercury compounds. The assistance of Mrs. Ebba Willstaedt, who carried out the activity determinations, is gratefully acknowledged.

Institute for Organic Chemistry and Biochemistry, University, Stockholm.

REFERENCES

1. v. EULER, H., and SVANBERG, O., Fermentsforsch. *3*, 330 (1919/20).
2. MYRBÄCK, K., Arkiv Kemi, Mineral. Geol. *8*, No. 29 (1923).
3. MYRBÄCK, K., Hoppe-Seylers Z. physiol. Chem. *158*, 160 (1926).
4. MYRBÄCK, K., and WILLSTAEDT, E., Arkiv Kemi *8*, 53 (1955).
5. MYRBÄCK, K., Arkiv Kemi *11*, 47 (1957).
6. MYRBÄCK, K., Festschrift Arthur Stoll, Sandoz AG, Basel 1957.
7. TORSSELL, K., Physiologia Plantarum *9*, 652 (1956).
8. TORSSELL, K., Arkiv Kemi *10*, 529 (1957).
9. GUZMAN BARRON, E. S., Advances in Enzymol. *11*, 201 (1951).
10. SMALT, S. M. A., KREKE, C. W., and COOK, E. S., J. Biol. Chem. *224*, 999 (1957).
11. MYRBÄCK, K., and WILLSTAEDT, E., Arkiv Kemi *11*, 275 (1957).

Tryckt den 22 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 53

Vorgelegt am 5. Juni 1957

Reduktonate und Pseudo-Reduktone

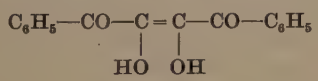
Ihr Verhalten gegen Oxydationsmittel

Von Hans von Euler und Hans Hasselquist

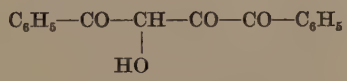
Wie wir in mehreren vorhergehenden Mitteilungen erwähnt haben (1), besteht neben den durch ihren Endiol-Gehalt gekennzeichneten, stark reduzierenden Reduktonen und Reduktonaten eine grosse Klasse von Substanzen — wir nennen sie Pseudo-Reduktone —, welche unter gewissen Bedingungen *ähnlich wie Reduktone reagieren, ohne Endiolgruppe zu enthalten.* Über solche Stoffe sind in neuester Zeit mehrfach Angaben gemacht worden, die insofern unzureichend sind, als das Verhalten dieser Stoffe gegen Oxydationsmittel, wie z. B. Dichlorphenol-indophenol (Tillmans-Reagens, TR), mit demjenigen der Reduktone nicht experimentell verglichen wurde. Es erscheint deswegen notwendig, die diesbezüglichen experimentellen Ergebnisse dieses Institutes schon jetzt mitzuteilen, bevor wir eine Zusammenstellung derselben auf Grund eines noch grösseren Versuchsmateriales folgen lassen.

Derivate des Benzoyl-formoins[1]

Aus der TR-Titration des Benzoyl-formoins berechnet sich für methanolische Lösung ein Endiol-Gehalt von rund 90 %. Diesem Umstand kommt die Formulierung I näher als die Ketoform II



I

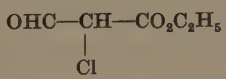


II

Es liegt nahe, aus der Endiolformel I des Benzoylformoins eine Halbacetalformel abzuleiten.

P. Karrer u. Mitarb. (Helv. 18 n. 19; 1935/36) fanden, dass der Jodverbrauch des Benzoylformoins in alkalischer Lösung dem eines Endiols nahezu gleichkommt.

Formyl-chlor-essigester



[1] Euler u. H. Hasselquist (2). Über die Umwandlung beim Schmelzen des gelben Benzoylformoins in die rote Endiolform siehe (3).

nach W. WISLICENUS (Chem. Ber. *43*, 3530, 1910) dargestellt, wurde in Form von Kristallen vom Schmpt. 85–90° erhalten und auf seine biologische Wirkung an Yoshida-Ascites-Tumor-Ratten untersucht.

Offenkettige Diketone

αα'-Diketoadipinsäure, Enolform Dihydroxymuconsäure und ihre Äthylester

Der αα'-Diketo-adipinsäure

$$COOH—CO—CH_2—CH_2—CO—COOH$$

entspricht das 1,4-Diketon

$$CH_3—CO—CH_2—CH_2—CO—CH_3$$

und ihre Dienolform

$$CH_3—C(OH)=CH—CH=C(OH)—CH_3$$

Von diesen leiten sich ab:
Acetonylaceton-dioxalsäure (vgl. (4))

$$CO_2H—CO—CH_2—CO—CH_2—CH_2—CO—CH_2—CO—CO_2H$$

resp. deren Ester L 63.

	Diketo-ad.säure	Dihydroxysäure	Dihydroxy-säure-ester
Schmpt.	100°	225—226°	160°
Löslichk.	leicht lösl. Wasser	schwer lösl. Wasser	schwer lösl. Wasser
Reakt. mit:			
TR		1 Mol	0,8 Mol
Tetrazoliumchl.			roter Niederschlag
$FeCl_3$	keine Farbenreakt.	intensiv rotbraune Färbung, rasch verblassend	Rotviolette Färbung, rasch verblassend

100 ml trockener Äther wurde mit 2,3 g feinverteilten Natrium und einer Mischung von 14,6 g Oxalsäure-diäthylester und 5,7 g Acetonylaceton versetzt, wobei von Zeit zu Zeit gekühlt wurde. Nach Ablauf der Reaktion liess man die Mischung bei 20° einen Tag stehen. Dabei fiel das Reaktionsprodukt als gelbes, kristallinisches Na-Salz aus, das getrocknet wurde. Das gelbe Kristallpulver wurde in 200 ml Wasser gelöst und langsam mit 4-n Schwefelsäure bis zur Kongoreaktion versetzt. Dabei fiel der Acetonylacetondioxalsäure-ester zuerst als Öl und dann als Kristalle aus (Ausbeute 9 g). Die nach Umkristallisieren hellgelben Nadeln schmolzen bei 102–103°.

Subst. Nr. L 63 ist leichtlöslich in Chloroform und Benzol, ziemlich löslich in Alkohol, löslich in warmen Wasser, unter Gelbfärbung. Nach Abkühlung verbraucht die Lösung sofort TR.

Titration der Sbst. L 63 mit TR. 7,1 mg Sbst. in 5 ml abs. Alkohol + 5 ml Wasser wurden mit 0.01-n TR versetzt. Sehr langsamer Verbrauch bei pH = 6. In Stickstoff-atmosph. wurde nun die Lösung mit 0,2 ml 2-n NaOH versetzt und mit TR weiter titriert. Verbrauch bis zum Umschlag (nach 45 Min.) 2,2 ml.

$$E_{\text{ber f. } C_{14}H_{14}O_8} = 157; \quad E_{\text{gef.}} = 323.$$

2,1 Mol Sbst. verbrauchen also 1 Mol TR.

Bromierung des Acetonylaceton-dioxalsäure-diäthylester (L 63)

310 mg Sbst. in 10 ml Chloroform gelöst und unter Abkühlung mit 0,05 ml Brom in 2 ml Chloroform versetzt, wobei das Brom (1 Mol Sbst. + 1 Mol Br_2) verbraucht wird. Die Chloroformlösung, in der sich stark HBr entwickelte, wurde im Scheidetrichter mit Wasser geschüttelt um HBr zu entfernen, dann mit wasserfreiem Natriumsulfat getrocknet und im Vacuum eingedunstet. Der Rückstand, ein gelbbraunes Öl, kristallisierte bald und wurde nach Verreiben mit Äthylacetat abfiltriert und mit Äthylacetat gewaschen. Aus dem Rohprodukt, 130 mg, wurden durch Umkristallisierung aus Äthylacetat gut ausgebildete citronengelbe Tafeln mit gelbgrüner Fluoreszenz gewonnen, die bei 149–150° unter Zers. schmolzen.

Sbst. 263

Analyse der Sbst. 263
Ber. für $C_{14}H_{16}O_8$ C 53,84 % H 5,17 %.
Gef. C 53,93 % H 5,55 %.

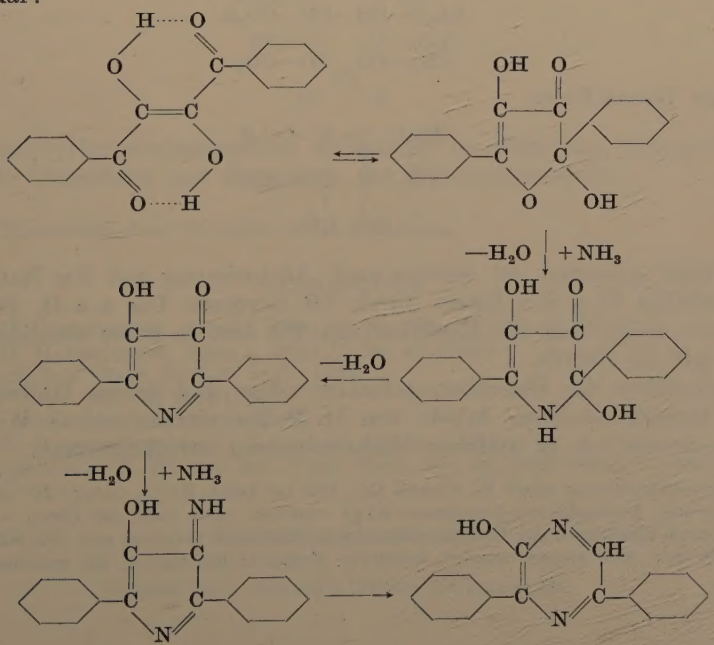
Einwirkung von Tetrazoliumchlorid auf den Acetonylaceton-dioxalsäureester

Mit 2,3,5-Triphenyl-tetrazolium-chlorid in Sodalösung ergab der Ester nach ca. 30 Min. eine deutlich rote Fällung, langsamer als Redukton oder Diketoadipinsäure. Entsprechender Unterschied des zeitlichen Verlaufes mit TR.

Bildungsschema des 2-Oxy-3,5-diphenyl-pyrazins (261)

In den vorhergehenden Mitteilungen (dieses Arkiv) wurde die Darstellung der Sbst. 261 aus Benzoyl-formoin und Ammoniak beschrieben und ausser der zur Zusammensetzung $C_{16}H_{12}O_5N_2$ führenden Analyse ((2), S. 412) die UV-Absorption in Äthanollösung angegeben ((1), Fig. 1).

Den Verlauf der Synthese des 2-Oxy-3,5-diphenyl-pyrazins stellen wir folgendermassen dar:



Nitrierung des 2-Oxy-3,5-diphenyl-pyrazins 261

500 mg der Sbst. 261 wurden in 5 ml konz. Schwefelsäure gelöst, wobei sich die Lösung gelbrot färbte. Nach Abkühlung auf 10° wurde portionsweise KNO_3 zugesetzt, im ganzen 1 g. Im Verlauf der Nitrierung wurde die Lösung citronengelb. Nach vollständigem KNO_3-Zusatz liess man die Lösung 2 Stunden stehen und verdünnte dann unter Kühlung vorsichtig mit Wasser. Das Nitrierungsprodukt fiel nun als gelbes Pulver aus, das abfiltriert, mit Wasser gewaschen und getrocknet wurde; Rohprodukt 200 mg

Nach zweimaligem Umkristallisieren aus Eisessig erhielten wir die Substanz 262 in reinster Form als kleine, intensiv gelbe Stäbchen, deren Schmpt. höher lag als 290°.

Sbst. 262

Die Sbst. 262 ist schwerlöslich in Wasser, Äthylacetat und Methanol, ziemlich löslich in Aceton mit intensiv grüner Fluoreszenz, leicht löslich in warmen Eisessig.

Analyse (338,3)

Gef.	C 57,87 %,	H 3,32 %,	N 16,22 %.
Ber. f. $C_{16}H_{10}O_5N_4$	C 56,82 %,	H 2,98 %,	N 16,56 %.

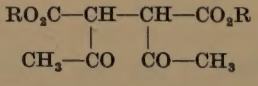
Es entstand also eine Dinitro-Verbindung nach der Gleichung:

$$C_{16}H_{12}O_5N_2 + 2\,HNO_3 \rightarrow C_{16}H_{10}O_5N_4 + 2\,H_2O$$

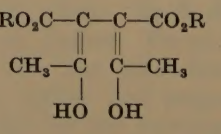
Auch bei der Nitrierung von 2,5-Diphenyl-pyrazins entsteht nach E. OCHIARI (J. Pharm. Soc. Japan *59*, 462, 1939) eine Dinitro-Verbindung[1].

Verzweigte, offenkettige Diketone und Dienole

Diacetbernsteinsäure-ester



und dessen Dienol-Form

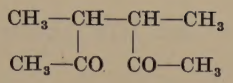


Wie früher gefunden (6) werden nach Alkalisierung mit 2-n NaOH in langsamer Reaktion 85 % des Esters durch TR oxydiert. Die a. a. O. Fig. 5 mitgeteilte Kurve zeigt, dass die Reaktion mit TR erst in stark alkalischer Lösung, bei etwa pH 12 eintritt.

Die Dienolform des Diacetbernsteinsäure-esters und dessen Halbenol-Form ist in einer bemerkenswerten Arbeit von H. P. KAUFMANN und G. WOLFF (7) besprochen, worauf wir in anderem Zusammenhang zurückkommen.

[1] *m*-Nitro-acetophenon nach R. CAMPS (5). 150 ml konz. HNO_3 unter –10° abgekühlt wird tropfenweise mit Acetophenon (im ganzen 25 g) versetzt, ohne dass die Temp. –8° überschritten wird. Durch Einführen der Reaktionslösung in Eiswasser scheidet sich das Nitroprodukt als gelbrotes Öl aus. Aus diesem werden hellgelbe Kristalle gewonnen, die gereinigt bei 80–81° schmelzen.

Das entspechende 2,3-Diacetyl-Butan



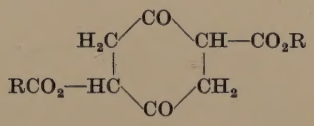
und dessen Endiolform wurden von uns folgenderweise über 3-Brom-butanon-2 dargestellt:

10 g 3-Brom-butanon-2 in 150 ml Äther gelöst, wurden mit 1,7 g feinverteiltem Natrium versetzt. Die Reaktion kommt unter Wärmeentwicklung sofort in Gang, wobei NaBr ausfällt. Die letzten Reste des noch nicht verbrauchten Natriummetalls wurden durch Erhitzen auf dem Wasserbad zur Reaktion gebracht. Nach Abfiltrieren des NaBr wurde die Lösung im Vacuum eingedunstet. Wir erhielten das 2,3-Diacetyl-butan mit einem Siedepunkt $Sp_{50} = 90–92°$. Die älteren Angaben sind: M. Démètre-Vladesco, Bull. Soc. Chim. *3*, 809 (1891):

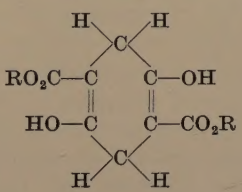
$Sp_{760} = 210°$. Ferner Ciamician u. Silber, Chem. Ber. *45* 1542 (1912): $Sp_{11} = 82°$.

2,3-Diacetyl-butan reduziert TR weder in saurer noch alkalischer Lösung.

Zyklische Diketone



Succinylbernsteinsäureester (Liebermann, Lieb. Ann. Chem. 404)



1 Mol. Succinylbernsteinsäureester verbraucht in alkalischer Lösung 0,97 Mole TR. Vgl. die Dienolform mit derjenigen der Diketoadipinsäure.

Stockholm, Universität, Inst. für organ.-chem. Forschung.

LITERATUR

1. Euler u. H. Hasselquist, Arkiv f. kemi *11*, Nr. 23 u. *11*, Nr. 44 (1957).
2. Euler u. H. Hasselquist, Arkiv f. kemi *11*, Nr. 43 (1957).
3. A. H. Blatt, Am. Chem. Soc. *58*, 1897; 1936.
4. Th. Gray, Chem. Ber. *33*, 1220 (1900).
5. R. Camps, Arch. d. Pharm. *240*, 5 (1902).
6. Euler u. H. Hasselquist, Arkiv f. kemi *11*, Nr. 23 (1957).
7. H. P. Kaufmann u. G. Wolff, Ber. dtsch. chem. Ges. *56*, 2523 (1923). — Lieb. Ann. *429*, 256 (1922).

Tryckt den 22 augusti 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB